Subcellular distribution of a neuronal nicotinic acetylcholine receptor in the rat brain

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Award date:
1993

Awarding institution:
University of Bath

Subcellular distribution of a neuronal nicotinic acetylcholine receptor in the rat brain

submitted by Frazer Ian Smillie

for the degree of PhD

of the University of Bath

1993

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For my wife, Ria.

Acknowledgements

I would like to thank my supervisors Prof George Lunt and Dr Sue Wonnacott for their guidance, advise and encouragement during the course of this degree and for their critical reading of this manuscript. I am grateful to RJ Reynolds for their financial support.

I am very grateful for the technical assistance provided by Colin Wilson in the Photography Department; Silvia Peters and Ursula Potter in the Electron Microscopy Unit; and by the entire Animal House crew. I would also like to thank Caron Billington for her contributions to the PAGE experiments, and especially Dr. Chris Boulton for his help and the loan of equipment. I would like to thank all my friends and colleagues, past and present, in the Nicotine Research Lab and in the departments of Biochemistry and of Pharmacology, especially Amber Elderfield, for their company, advice, comments and support.

Finally, I would like to thank my parents and family, my friends, and especially my wife and son for their support.

Summary

The aim of this project was to use immunological techniques to determine the subcellular distribution of the α4ß2 subtype of neuronal nicotinic acetylcholine receptor (nAChR) in the mammalian brain. Three approaches were taken:-

1) Immunoelectron microscopy of isolated presynaptic terminals (synaptosomes)

2) An attempt to immunoisolate synaptosomes bearing the α4ß2 subtype, leading to synaptosome characterization studies to determine neurotransmitter synthesizing enzyme content.

3) Pharmacological and immunological characterization of subcellular fractions.

Monoclonal antibodies directed against the extracellular domain of detergent solubilized α4ß2 were donated by J. Lindstrom and polyclonal sera directed against the cytoplasmic region between transmembrane regions M3 and M4 of various subunit subtypes were donated by S. Rogers.

The results indicate the viability of all three approaches, although an extensive immunoelectron microscopy study would be required, because of the low α4ß2 concentration. Immunoelectron microscopy and immunoisolation experiments were compromised by an unanticipated immunological recognition problem and so could not be pursued to completion. A pharmacological and immunological characterization of subcellular fractions reinforced previous studies indicating that a significant proportion of the α4ß2 subtype was located at the presynaptic terminal.

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Abbreviations

ACh - acetylcholine

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

5AS - 5-aminosalicylic acid

BSA - Bovine Serum Albumin

Bicine - N,N-bis(2 hydroxyethyl) glycine

CNS - central nervous system

DAB - 3,3'-Diaminobenzidine

DMSO - Dimethyl sulfoxide

EDTA - Diaminoethane tetra-acetic acid, disodium salt

ELISA - Enzyme-linked immunosorbent assay

GABA - γ-aminobutyric acid

HEPES - N-(2-Hydroxyethyl) piperazine-N-(2-ethane sulfonic acid)

5-HT - 5-hydroxytryptamine

LDCV - large dense-core vesicles

LDH - Lactate dehydrogenase

LGIC - ligand-gated ion channels

mAb - monoclonal antibody

mepps - miniature end-plate potentials

MOPES - 3-(N-morpholino) propanesulphonic acid

nAChR - nicotinic acetylcholine receptors

NADH - ß-Nicotinamide adenine dinucleotide, reduced form

NMDA - N-methyl-D-aspartic acid

PAGE - polyacrylamide gel electrophoresis

PBS - Phosphate Buffered Saline

PEI - polyethleneimine

pnpp - paranitrophenyl phosphate

PIPES - piperazine-N,N'-bis(2-ethanesulphonic acid)

PMSF - Phenylmethylsulfonyl fluoride

SDS - sodium dodecyl sulphate

SJC - synaptic junctional complexes

SPM - synaptic plasma membranes

SSV - Small synaptic vesicles

TBS - Tris buffered saline

Tris - Tris (hydroxymethyl) amino methane

TEMED - N,N,N',N'Tetramethyl ethylenediamine

TMB - Tetramethyl benzidine

Tissue Preparation Abbreviations

H - Homogenate, homogenized brain tissue (section 3.2.1)

Hb - Synaptosome preparation, Homogenate fractionated using a 2-step sucrose gradient (Rapier *et al*, 1988, section 3.2.2)

S1 - Supernatant fraction from low speed centrifugation (Gray and Whittaker, 1962; section 3.2.2)

P2 - Plasma membrane preparation, Pellet from medium speed centrifugation of S1 (Gray and Whittaker, 1962; section 3.2.2)

P2b - Synaptosome preparation, P2 fractionated using a 2-step sucrose gradient (Gray and Whittaker, 1962; section 3.2.2)

Fx - Fractions 1-5, S1 fractionated using a 4-step Percoll gradient (Dunkley *et al*, 1987, 1988; Harrison *et al*, 1988; section 3.2.4)

"non sunt multiplicanda entia praeter necessitem"

[entities should not be multiplied beyond necessity] -

"Occam's razor" William of Occam (1285-1349)

obtained from Durand de Saint Pourçain (1270-1334).

Chapter 1 Introduction

1.1 The chemical synapse

In the human brain there are approximately 1000 different cell types including 10^{11} neurons. Every neuron has, on average, 1000s of connections with other neurons, muscle cells or secretory cells. The classic picture of the neuron and intraneuronal signal propagation is summarized in figure 1.1. The modern model of propagation is slightly modified, to account for signal propagation in all neuronal regions, by including the movement of other ions especially Ca^{2+} and Cl^- (reviews: Armstrong, 1992; Catterall, 1992; Hille, 1992; Neher, 1992; Sakmann, 1992; and Stevens, 1993). Some interneuronal connections are electrical (Bennet *et al*, 1991; Makowski *et al*, 1977, 1984), but the vast majority are made via chemical synapses.

The ultrastructure of the chemical synapse was determined by electron microscopy (1953, Sjöstrand; review: Jones, 1975), the classic description of synaptic structure and function is summarized in figure 1.2. Transmission at chemical synapses is significantly slower than at electrical synapses, but they have several advantages (Hall and Sanes, 1993), they:-

i) permit signal amplification and so avoid attenuation problems.

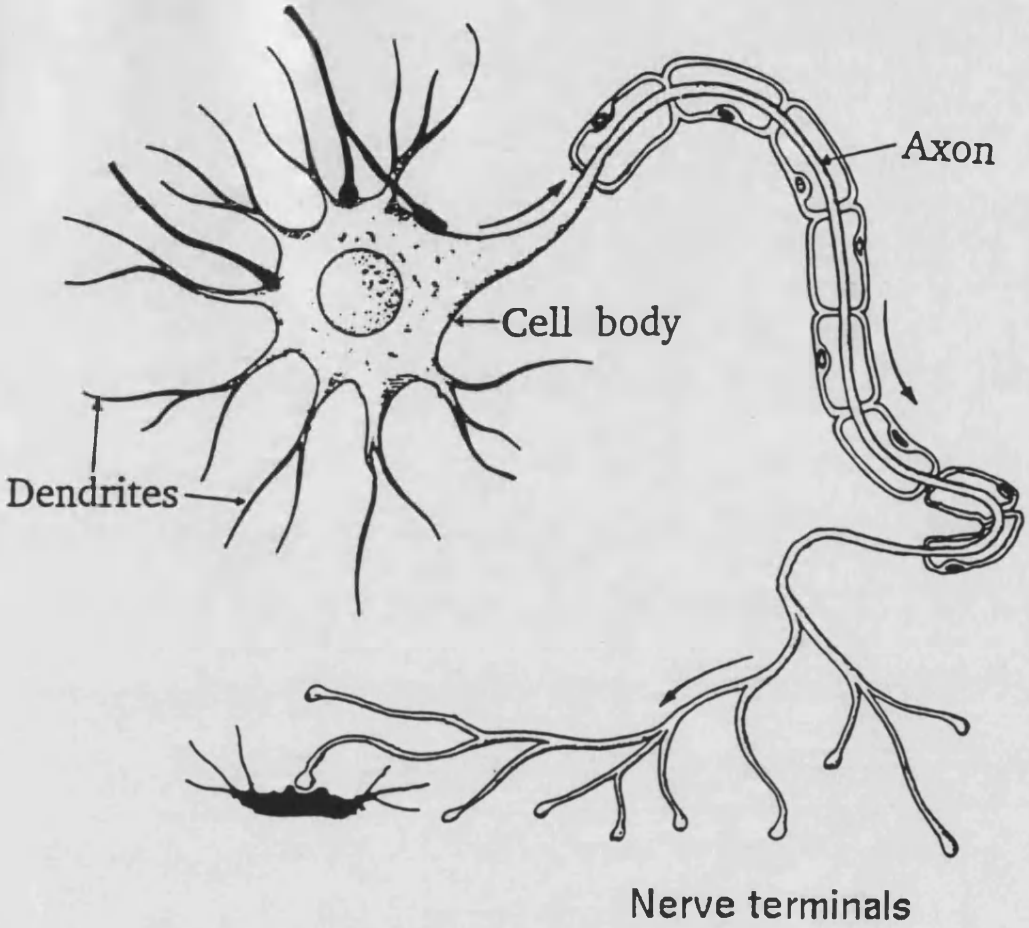
ii) allow easy conversion between excitatory and inhibitory signals.

iii) provide multiple regulation levels.

iv) allow multiple responses by the interplay of transmitter and receptor types.

v) fulfil the minimum requirements of regulation and feedback to be individually regarded as the fundamental units of information processing and storage (Hebb, 1949).

Figure 1.1 The neuron



Deitres first described the neuron in 1865. It consists of multiple branching dendrites and a single axon converging on a nucleated soma. Ramó n y Cajal demonstrated that neurons are discrete units through which information flows unidirectionally. A signal propagates as described by the ionic hypothesis (Hodgkin, Huxley and Katz, 1949; Hodgkin and Huxley, 1952) from the postsynaptic junction on multiple branching dendrites and the soma (input), down the axon (long range transmission, up to 1m) to presynaptic terminals (output). The term synapse was originally introduced by Sherrington in 1897 (historical review: Changeux, 1983).

Figure 1.2 The chemical synapse

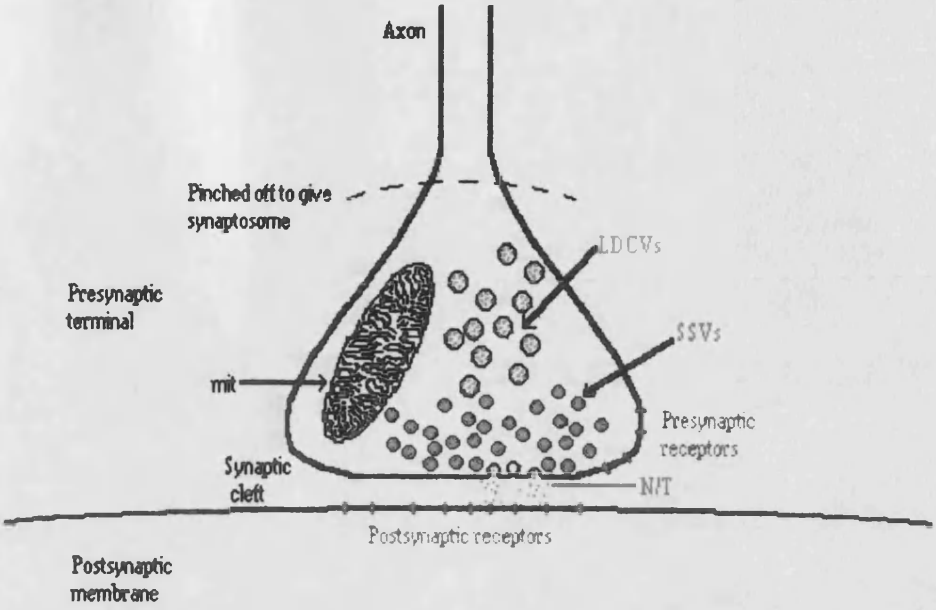


Figure 1.2 is not to scale. The chemical synapse functions (chemical transmitter hypothesis, 1935, Dale) by the transmission of a chemical signal from synaptic terminal (500nm diameter) across the synaptic cleft (12nm) to the postsynaptic surface. The neuroactive substances are stored in and released from synaptic vesicles (De Robertis and Bennett, 1955), each vesicle represents one quantum (quantal release hypothesis, Del Castillo and Katz, 1954) for random (miniature end-plate potentials, mepps: Fatt and Katz, 1950, 1952) or calcium induced release (calcium hypothesis, 1969, Katz). Small synaptic vesicles (SSV, uniform diameter, 50nm) contain classical small molecule neurotransmitters (Südhof and Jahn, 1991), large dense-core vesicles (LDCV, heterogeneous diameter, around 100nm) contain neuromodulatory peptides and proteins and bioamines (Sossin *et al*, 1989). Neurotransmitters interact with receptor molecules on the post-synaptic cell causing membrane depolarization (excitatory) or hyperpolarization (inhibitory) (Eccles, 1963). An action potential is initiated in the post-synaptic cell as a result of temporal and spacial summation synaptic responses. Synaptosomes are formed by "pinching off" presynaptic terminals at the neck. They often contain mitochondria (mit) and so are metabolically active. The functional role of presynaptic receptors is explored in the text.

There are over 100 neuroactive substances involved in interneuronal communication, they include the classical neurotransmitters and a variety of substances which modulate the response to or the release of these neurotransmitters (figure 1.2): a small selection neuroactive substances are released at any particular synapse (Jessel and Kandel, 1993; McGeer *et al*, 1987). There are 2 types of vesicles present at nerve terminals with different modes of storage and release (Kelly, 1993), both bud from the trans Golgi apparatus within the cell body and are transported along the axon to the nerve terminals:-

a) The SSVs are filled (McMahon and Nicholls, 1991) at the terminal to 100mM concentrations (approximately) of either acetylcholine, glutamate, γ-aminobutyric acid (GABA), glycine or one of the biogenic amines. When an action potential reaches the terminal voltage-gated Ca^{2+} channels open in addition to the Na^{+} channels (Fox *et al*, 1987; Nowycky *et al*, 1985; review McMahon and Nicholls, 1991). The resulting Ca^{2+} influx raises the intracellular Ca^{2+} concentration to 200-300μM in microdomains in the vicinity of neurotransmitter release sites (Llinás *et al*, 1992), which dramatically increases the probability of neurotransmitter release above the basal level (mepps review: Stevens 1993). The Ca^{2+} induced release is very rapid, within 1ms of Ca^{2+} influx there is a 1ms neurotransmitter efflux (Lim *et al*, 1990; Lindau *et al*, 1992), which empties 10% of the docked vesicle pool present in the active zones (Cohen *et al*, 1991; Smith and Augustine, 1988), ie 0.1% of the total vesicle pool. After neurotransmitter release SSVs can be recycled via (nerve terminal) endosomes and/or rapidly refilled with fresh neurotransmitter at the terminal (Südhof and Jahn, 1991). (Neurotransmitter release reviews:- Almers and Tse, 1990; Kelly, 1993; McMahon and Nicholls, 1991; Sihra and Nichols, 1993; Südhof and Jahn, 1991). The mechanism for

calcium independent neurotransmitter release is unclear, it may merely involve the release of intracellular calcium stores (Adam-Vizi, 1992).

b) The LDCVs are less common than SSVs and cannot be refilled at nerve terminals, but must return to the Golgi (Sossin *et al*, 1989). The Ca^{2+}-induced exocytosis of LDCVs is slower (50ms) and occurs at different sites (Pow and Morris, 1989; Thureson-Klein, 1983; Thureson-Klein and Klein, 1990) to release from SSVs, probably via an independent release mechanism (Jessel and Kandel, 1993; Kelly, 1993).

Before the breakdown and/or re-uptake of neuroactive substances (Iversen, 1979) they might interact with receptors on the pre- or the post synaptic membranes. Presynaptic receptors are typically metabotropic (Bourne and Nicoll, 1993). They regulate the action potential modulation of Ca^{2+} influx and neurotransmitter release, in response to transmitter released by the terminal itself (autoreceptors) or other terminals (heteroreceptors).

Receptors fall into two categories:-

a) Metabotropic receptors (Ross, 1989) are coupled via GTP-Binding (G-) proteins to effectors, which enzymically produce intracellular second messengers. The metabotropic receptors are a large class, they include families of receptors, each of which slowly respond (ie persist seconds or minutes) to one the small molecule neurotransmitters (eg the muscarinic acetylcholine receptor family respond to acetylcholine [ACh]) either to open or close ion channels or to regulate and modify cellular proteins in coordinated responses (reviews: Bourne and Nicoll, 1993; DeVries and Baylor, 1993; He and Rosenfeld, 1991; Ross, 1989).

b) Ionotropic receptors are ligand-gated ion channels (LGIC) which span the plasma membrane, when the relevant small molecule neurotransmitter binds to the extracellular recognition sites long range conformational changes cause the rapid opening of a central pore (see nicotinic acetylcholine receptors [nAChR], section 1.2,1.3,1.4). The ion flux through this pore is transient before closure and LGICs produce rapid synaptic potentials lasting only milliseconds (Sakmann, 1992). Like metabotropic receptors, a family of LGICs are expressed to recognize each of the small molecule neurotransmitters, the pharmacology and functional behaviour of each receptor subtype varies, providing a wide range of responses to different conditions (sections 1.2,1.3). The LGICs are divided on the basis of sequence identity and structural similarity into 2 superfamilies:-

i) the glutamate receptors [Gasic and Hollmann, 1992; Sommer and Seeburg, 1992]: NMDA (N-methyl-D-aspartic acid), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate classes

ii) nAChR (section 1.3), serotonin/5-HT (5-hydroxytryptamine), GABA (Wisden and Seeburg, 1992) and glycine (Betz, 1992) receptors

There is little sequence similarity, but some structural similarity between the 2 superfamilies (Unwin 1993a). The LGICs are also compared with the porin channel, which is non-selective to ions, and the voltage gated channels, which are far more ion selective than LGICs (Jessel and Kandel, 1993). The LGICs are charge specific, they can be: either cation selective (depolarizing, tending to an excitatory synaptic potential), eg ACh, 5-HT and glutamate receptors; or anion selective (hyperpolarizing, tending to an inhibitory synaptic potential), eg glycine and $GABA_A$ channels (Sakmann 1992; Unwin, 1993a).

1.2 The composition and structure of nAChRs

The nAChRs are the best characterized family of LGICs for a variety of historical reasons:-

a) the high concentration of a neuromuscular form of nAChRs in the electric organs of the electric fish *Torpedo*, *Narcine* and *Electrophorus electricus*, which allowed their purification during the early 1970's (Changeux, 1981; Conti-Tronconi and Raftery, 1982),

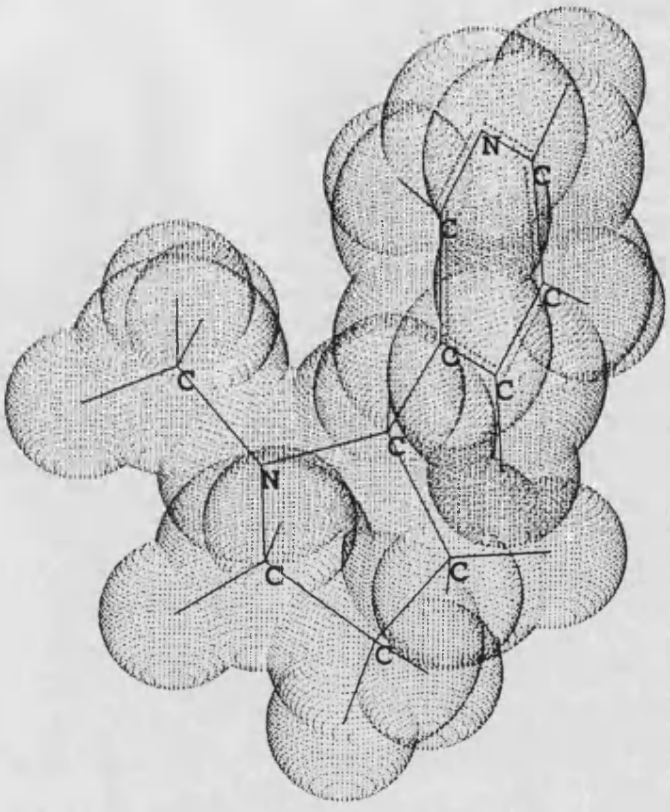
b) the abundance of the neuromuscular form of nAChR in mammalian muscle relative to the neuronal forms found in the brain (Changeux, 1981; Karlin 1990),

c) the early discovery of natural neurotoxins, especially the snake toxins such as α-Bungarotoxin from *Bungarus multicinctus*, which were used as a pharmacological probes (Lee, 1979; Wonnacott, 1987b) and affinity ligands (Norman *et al*, 1982; Wonnacott *et al*, 1982),

d) the autoimmune disease myasthenia gravis, which is characterized by the production of antibodies which recognize the muscle nAChR (Erlanger *et al*, 1984; Vincent, 1980). The antibodies produced in this disease initiated the immunological investigation of the nAChRs (Lindstrom, 1984; Lindstrom *et al*, 1987, 1991). Neuronal nAChR levels are abnormal in important diseases including Alzheimer's and Parkinson's disease (Aubert *et al*, 1992; Giacobini, 1992; Kellar and Wonnacott, 1990; Lange *et al*, 1991; Perry *et al*, 1987).

e) nicotine (figure 1.3), the agonist which defines the family, is the major active component amongst the 1000s of tobacco smoke ingredients: it causes a variety of psychoactive effects (Balfour, 1982; Clarke, 1987; Wonnacott *et al*, 1990b) including addiction (Benowitz, 1988; Pommerleau, 1984). The drug is very

Figure 1.3 Nicotine



Nicotine is the agonist which defines the nicotinic acetylcholine receptors. The molecular structure is shown in figure 1.3.Note the chiral centre, there are + and - isomers, the latter has far greater psychoactive and pharmacological potency (see references to smoking and addiction in the text). Produced using Hyperchem for Windows - Autodesk.

popular, at present tobacco is consumed by one third of the population of most western countries and extensively worldwide, prompting considerable and continuous interest in its effects upon the brain and the body (Bock and Marsh, 1990; Clark, 1991; Lippiello *et al*, 1992).

In 1980 Raftery *et al* published the results of N-terminal amino acid sequence analysis of each *Torpedo* subunit, the corresponding oligonucleotide probes were used to clone and sequence the entire subunit genes (Ballivet *et al*, 1982; Noda *et al*, 1982, 1983a; Sumikawa *et al*, 1982) initiating extensive cross hybridization, cloning and immunopurification studies, which have reported nAChR subunit families in several species (Deneris *et al*, 1991; Lindstrom *et al*, 1987; Luetje *et al*, 1990a; Role, 1992; Sargent, 1993). There are 5 homologous (Noda *et al*, 1983b) subunit subtypes ($\alpha 1$, $\beta 1$, γ, δ, ϵ), which are expressed in the 2 neuromuscular forms of nAChRs:-

i) $\alpha 1_2 \beta 1 \gamma \delta$, extrasynaptically or at developing synapses and

ii) $\alpha 1_2 \beta 1 \epsilon \delta$, at mature synaptic junctions (Hall and Sanes, 1993)

The neuronal nAChRs, which have been discovered, are composed either entirely of α subunits (rat:-$\alpha 2$ to $\alpha 7$; chick:- $\alpha 2$ to $\alpha 8$) or of α subunits and β subunits ($\beta 2$ to $\beta 4$) (Deneris *et al*, 1991; Sargent, 1993). The subunits are homologous (Bossy *et al*, 1988; Cockcroft *et al*, 1992; Deneris *et al*, 1991; Sargent, 1993), but there is greater sequence similarity within subunit classes than between subunit classes. The α subunits are distinguished by a pair of consecutive cysteines at 192 and 193 (*Torpedo* amino acid numbering), which are in the vicinity of the ligand binding site (Kao *et al*, 1984). Other affinity labelling experiments indicate that the acetyl

group binds close to the cysteine pair on the α subunits (Galzi *et al*, 1991a), where the quaternary ammonium binds is unclear (Unwin, 1993a), but it may well be to a negative subsite formed at least partly by acidic residues on the adjacent subunit (Unwin, 1993a). Galzi *et al* (1991a) demonstrated that there are at least two acetylcholine binding sites per receptor and the associated α subunits are not adjacent. The ß subunits were originally defined by the ability of ß2 to substitute for ß1 in functional receptors (Deneris *et al*, 1988). The ß subunits may influence numerous receptor properties (Cachelin and Jaggi, 1991; Papke and Heinemann, 1991). The α4ß2 neuronal nAChR subtype has been shown to be a pentameric (Anand *et al*, 1991; Cooper *et al*, 1991) hetero-oligomer, and the muscle form also appears to be pentameric (above), but there is no evidence that any other neuronal nAChR is pentameric, although this extrapolation is frequently suggested. The subunit composition of neuronal nAChRs varies:

a) A single α subunit subtype may be expressed, eg oocyte expression combined with whole cell recording studies confirm that α7 (Couturier *et al*, 1990a), and perhaps α4 (Deneris *et al*, 1991) can be expressed as functional multimeric homo-oligomers,

b) A pair of subunits, one α and one ß subunit subtype may be co-expressed, eg α2, α3, α4 have been co-expressed in pairwise combinations with ß2 and ß4 subunits as functional multimeric hetero-oligomers ie α2ß2, α3ß2, α4ß2, α2ß4, α3ß4, α4ß4 (Ballivet *et al*, 1988; Boulter *et al*, 1987; Couturier *et al*, 1990b; Deneris *et al*, 1988; Duvoisin *et al*, 1989; Wada *et al*, 1988; review Deneris *et al*, 1991). There is some evidence that receptors with different stoichiometries can sometimes be found or expressed, eg the α4ß2 subtype, which was characterized in early experiments seemed to be composed of 2:3 α4:ß2 subunits (Anand *et al*,

1991; Cooper *et al*, 1991), but there is some evidence that a $\alpha 4_3 ß2_2$ can be expressed, which has different conductance characteristics (Papke *et al*, 1989).

A pair of α subunits subtypes may be co-expressed. Immunoprecipitation experiments indicate $\alpha 3\alpha 5$ and $\alpha 4\alpha 5$ combinations can be expressed *in vivo* (Conroy *et al*, 1992), and antisense oligonucleotide deletion experiments indicate the *in vivo* coassembly of $\alpha 3\alpha 7$ (Listerud *et al*, 1991) and $\alpha 7\alpha 8$ (Schoepfer *et al*, 1990) combinations.

c) More than 2 subunit subtypes can be expressed, providing some αs are included, eg the *in vivo* expression of single multimeric hetero-oligomers containing:- $\alpha 3$, $\alpha 5$ and ß4 (Vernallis *et al*, 1993); and $\alpha 3$, $\alpha 4$ and $\alpha 5$ (Conroy *et al*, 1992) have been demonstrated by immunoprecipitation experiments.

Each subtype combination has distinctive pharmacological and conductance characteristics (MacAllan *et al*, 1988; Luetje *et al*, 1990b; Deneris *et al*, 1991; Sargent, 1993) which are being evaluated (eg Amar *et al*, 1993). Despite the bewildering array of functionally expressed receptors, co-expression seems neither random nor non-specific: there are many subunit subtype combinations which do not produce functional receptors (Deneris *et al*, 1991) and a single neuron may express more than one receptor combination, yet maintain receptor segregation (Vernallis *et al*, 1993).

The 3-dimensional appearance of the acetylcholine receptor is increasingly being resolved (now 9Å: Unwin, 1993b) by electron microscopy of semicrystalline arrays of the *Torpedo* electric organ form (Heuser and Salpeter, 1979). This resolution

is inadequate to solve the nAChR structure, but the information gained does contribute to the experimental verification of theoretical topographical models (Unwin, 1993a). Hydropathy plots (Kyte and Doolittle, 1982) indicate that all nAChR subunits, and indeed all LGICs, have 4 significant hydrophobic stretches which are thought to span the plasma membrane, from the extracellular N-terminal end they are named M1-M4 (reviews: Changeux *et al*, 1992b; Connolly, 1989; Galzi *et al*, 1991b; Guy and Hucho, 1987; Unwin, 1993a). Various methods have been employed to establish topography, the present consensus favours the model displayed in figure 1.4 for each subunit of the entire receptor.

The transmembrane region can be analyzed in greater detail. There is increasing evidence (Ortells and Lunt, 1993, in preparation) that the membrane spanning regions M1, M3 and M4 are not α-helical, Unwin (1993a) suggests they form a continuous rim of ß-sheet as has been found in the solved structures of 2 pentameric toxin complexes (Sixma *et al*, 1991; Stein *et al*, 1992). There is considerable evidence to indicate that the major contributor to the pore lining is the M2 region. Imoto *et al* (1986) used single-channel conductance measurements (Neher *et al*, 1978; Neher and Sakmann, 1976) of receptors composed of chimeric subunits to demonstrate that the M2 region determined channel conductance. Labelled channel blockers penetrate the open pore and bind to M2 residues (Galzi *et al*, 1990; Giraudat *et al*, 1986; Hucho *et al*, 1986; Leonard *et al*, 1988; Revah *et al*, 1990). Site-directed mutagenesis of the M2 region can affect:- the binding of channel blockers (Changeux *et al*, 1992a; Charnet *et al*, 1990; Leonard *et al*, 1988), channel conductance (Imoto *et al*, 1988; Villarroel *et al*, 1991; Villarroel and Sakmann, 1992), channel selectivity (Galzi *et al*, 1992) and

Figure 1.4 The favoured model for nAChR topography

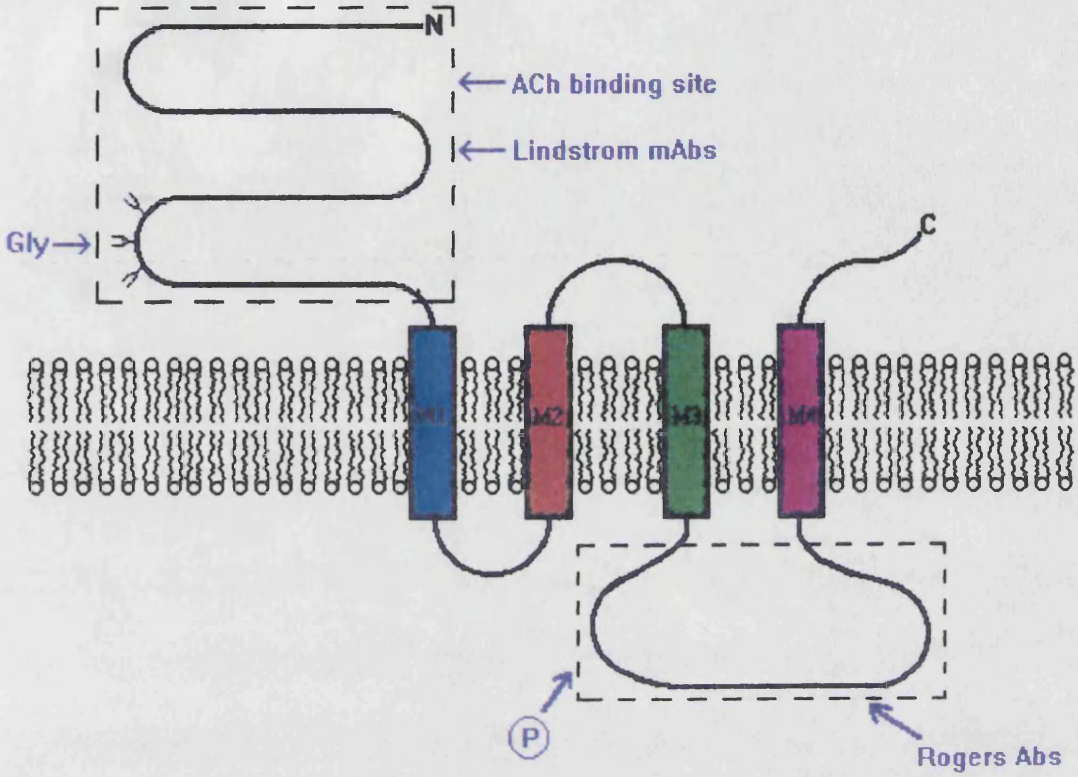


Figure 1.4 displays the 4-helix folding model (Noda *et al*, 1983; Devillers-Thiéry *et al*, 1983; Claudio *et al*, 1983), now favoured (Galzi *et al*, 1991; Changeux *et al*, 1992; Unwin, 1993) over a variety of models suggested in the 1980's (Popot and Changeux, 1984; Ratman *et al*, 1986a; McCrea *et al*, 1987). The acetylcholine (ACh) binding site, the glycosylation (Gly) sites (Popot and Changeux, 1984) and the main immunogenic region (Lindstrom, 1984; Ratnam *et al*, 1986b) are located in the extracellular N-terminal region. The polypeptide spans the membrane at regions M1-M4. Phosphorylation (P) sites (Huganir *et al*, 1986; Swope *et al*, 1992) and the amphipathic region (Dwyer, 1988; Roth *et al*, 1987) are located in the M3-M4 cytoplasmic loop, the C-terminus is extracellular (McCrea *et al*, 1987).

pharmacological properties, eg channel desensitization (Bertrand *et al*, 1992; Revah *et al*, 1991). The majority of these experiments indicate that the secondary structure of the M2 region is α-helical (Changeux *et al*,1992a, b; Galzi *et al*, 1991b; Unwin, 1993a) and ion flux is controlled by the rings of amino acids which face the lumen (Changeux *et al*, 1992b; Charnet *et al*, 1990; Galzi *et al*, 1991a, b, 1992; Imoto *et al*, 1988; Konno *et al*, 1991; Pedersen *et al*, 1992; Villarroel and Sakmann, 1992). Figure 1.5 displays a possible arrangement for the membrane spanning region of the receptor. The control of channel opening is not yet understood (Changeux *et al*, 1992b; Galzi *et al*, 1991b; Unwin, 1993a), but it seems to involve the highly conserved M1 region (Lo *et al*, 1991).

1.3 Pharmacology and modulation of the nAChR

Ligands bind to receptors and induce (agonist) or block (antagonist and channel blocker) or modulate (below) a functional response. Each new functional nAChR combination is pharmacologically characterized (see subunit composition, section 1.2), and there is a continuous effort to find and characterize new ligands (eg methyllycaconitine: Jennings *et al*, 1986; MacAllan *et al*, 1988; Ward *et al*, 1990), and ligand derivatives (eg anatoxin-a derivatives: Wonnacott *et al*, 1992). Ligand binding (review: Lunt, 1987; Yamamura *et al*, 1985) can exploit subtle differences between subunit subtypes to distinguish receptor combinations (Clarke, 1992; Deneris *et al*, 1991; Luetje *et al*, 1990b; MacAllan *et al*, 1988; Wonnacott, 1987). The receptor combinations responsible for high affinity [^{3}H]-nicotine binding are of particular interest, because of the wealth of localization and pharmacological

Figure 1.5 Diagrammatic representation of the distribution of the membrane spanning hydrophobic regions of the nAChR

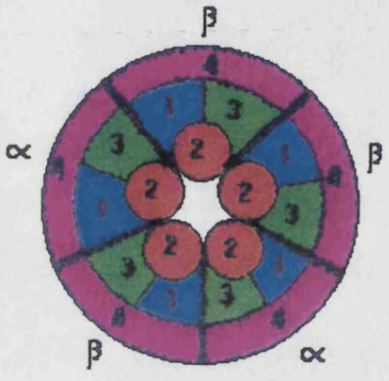


Figure 1.5 is a diagrammatic representation demonstrating a possible distribution of the hydrophobic regions M1-M4 as they span the membrane, based on Unwin (1993a). The M2 region is probably α-helical and pore lining, the M4 region seems to be facing the lipid and M1, M3 and M4 are of undetermined secondary structure. The 2 α subunits are not adjacent. Kuhse *et al* (1993) review the assembly of pentameric LGICs.

(ie competitive binding, etc) data using this ligand (section 1.4). The $\alpha4_2ß2_3$ is mainly, if not wholly (>90%), responsible for high affinity [^{3}H]-nicotine and the high affinity [^{3}H]-cytisine binding (Flores *et al*, 1992; Pabreza *et al*, 1991; Nakayama *et al*, 1991; Whiting *et al*, 1987a, b; Whiting and Lindstrom, 1986a, 1987, 1988). Two isoforms of the rat α4 are expressed (α4-1 and α4-2) due to splicing variation during transcription, however all evidence to date indicates that the two forms of α4ß2 are identical in terms of distribution, pharmacological properties, functional responses and immunological detection with the antibodies used in this project, (Connolly *et al*, 1992).

Receptor modulation and regulation may be homospecific (self regulation) or heterospecific (trans-regulation by other receptors) and can also be divided into receptor turnover (Hollenberg, 1985a) and receptor modification (Hollenberg, 1985b). Examples of receptor modification include:-

a) Prolonged agonist exposure results in reversible receptor desensitization (Katz and Thesleff, 1957), first to an intermediate desensitized state (I), then over a longer time period to a fully desensitized state (D) (see Changeux *et al*, 1992b; Changeux and Revah, 1987).

b) Certain hormonal steroids, eg progesterone, appear to bind to nAChRs and reversibly modify the receptor to inhibit ACh evoked responses, this modulation does not involve channel blocking, desensitization or competitive inhibition (Bertrand *et al*, 1991; Valera *et al*, 1992).

c) Calcium ions bind to some neuronal nAChRs at an extracellular site and at physiological concentrations potentiate ACh evoked ion flux (Mulle *et al*, 1992b; Vernino *et al*, 1992). Some neuronal nAChRs are permeable to calcium (Mulle

et al, 1992a; Vernino *et al*, 1992) and this allows the intracellular modulation which calcium ions can initiate (Mulle *et al*, 1992a), possibly including neurotransmitter release (Wonnacott *et al*, 1990a) and receptor regulation (DeLorme and McGee, 1988; Mulle *et al*, 1992a).

d) Several agents, eg Substance P (Margiotta and Berg, 1986; Simmons *et al*, 1990) are known heterospecifically to modulate receptor activity by changing its tendency to desensitization via second messenger systems (Changeux, 1981). Often nAChR phosphorylation is changed: desensitization increases with increasing phosphorylation of the M3-M4 transmembrane section (Huganir and Greengard, 1990; Steinbach and Zemple, 1987; Swope *et al*, 1992).

Examples of regulation of receptor expression include:-

a) Metabotropic receptor regulation of receptor expression via second messenger systems (Bourne and Nicoll, 1993; DeVries and Baylor, 1993; He and Rosenfeld, 1991; Ross, 1989).

b) The atypical agonist-induced upregulation of the $\alpha 4\beta 2$ receptor which is observed in some brain regions after chronic exposure (Flores *et al*, 1992; Hagino and Lee, 1985; Kellar *et al*, 1989; Marks *et al*, 1983, 1986; Martino-Barrows and Kellar, 1987; Pauly *et al*, 1991; Sanderson *et al*, 1993). The high affinity agonist binding to the desensitized form of the receptor (Changeux *et al*, 1992b; Changeux and Revah, 1987) may be the trigger (Schwartz and Kellar, 1985; Wonnacott, 1990) for this upregulation. The functional significance of upregulation remains a contentious issue, some groups suggest that chronic agonist exposure causes the numbers of functional receptors to fall (Hillard and Pounds, 1993; Hulihan-Giblin *et al*, 1990; Lapchak *et al*, 1989; Lukas, 1991; Marks *et al*, 1985), whilst others

show an upregulation of functional response (Clarke *et al*, 1988; Fung, 1989; Ksir *et al*, 1987; Rowell and Wonnacott, 1990; Wonnacott *et al*, 1990a). These results may reflect differences in:-

i) experimental protocol (eg mode of agonist delivery)

ii) brain region

iii) agonist type

iv) receptor type, ie other receptors may (Harsing *et al*, 1992; Stolerman *et al*, 1992) or may not (Grady *et al*, 1992) contribute to the functional response.

At present, it is difficult to resolve the issue, because no available technique can independently confirm the functional activity of the $\alpha 4\beta 2$ receptor population in a particular tissue (reviews: Drasdo, 1993; Sanderson, 1993).

c) Other chemicals, which are normally present in the brain (eg neuronal growth factor: Rogers *et al*, 1992; Whiting *et al*, 1987c), or are introduced by consumption (eg ethanol: Yoshida *et al*, 1982), regulate nAChR expression.

1.4 Immunology and localization of nAChRs

For historical reasons (section 1.2), immunological probes were raised against nAChRs before any other LGIC. Affinity and immunoaffinity purification of detergent solubilized nAChRs allowed the production and screening of immunological probes (Lindstrom, 1984; Lindstrom *et al*, 1987, 1991). This approach proved very powerful and immunological cross-reaction allowed antibodies to be raised against different receptor subunits from a variety of species (Lindstrom *et al*, 1987, 1991). Epitope mapping was achieved using

immunological protection techniques (Tzartos *et al*, 1981; Tzartos and Lindstrom, 1980) and peptide fragment analysis (Maelicke *et al*, 1989). Anti-idiotypic antibodies have been raised and show anti-nAChR binding (Lippiello *et al*, 1991), which seems to include $\alpha 4\beta 2$ recognition (Fluhler *et al*, 1992). More recently, as subunits have been sequenced, it has been possible to raise antibodies against specific receptor regions: the M3-M4 intracellular region is popular, because it is relatively accessible and has little sequence similarity between subunit subtypes (Flores *et al*, 1992; Hill *et al*, 1993; Rogers *et al*, 1991b). Anti-nAChR antibodies have been used to immunoisolate receptors, which can then be pharmacologically (Flores *et al*, 1992; Whiting and Lindstrom, 1986a), immunologically (Whiting *et al*, 1987b) and structurally (Maelicke *et al*, 1989; Whiting and Lindstrom, 1986b, c) characterized.

Some anti-nAChR antibodies have been used for immunohistochemical studies of brain slices to localize the regional distribution of receptor subunits subtypes: the antibodies were fluorescence labelled (Fluhler *et al*, 1992; Lippiello *et al*, 1991), radiolabelled (Swanson *et al*, 1987), enzymically labelled (Deutch *et al*, 1987; Hill *et al*, 1993) and colloidal gold labelled (Hill *et al*, 1993). These experiments have been paralleled by mRNA hybridization (Daubas *et al*, 1990; Morris *et al*, 1990; Wada *et al*, 1989, 1990) and radioligand binding (eg acetylcholine, nicotine and α-bungarotoxin: Clarke *et al*, 1985) to brain slices. Radioligand binding provides pharmacological information which can be used to localize those receptor isoforms which can be distinguished (section 1.3), this information contrasts with that from the former two techniques:- the distribution of subunit subtypes only gives limited information about the distribution of

receptor isoforms, because so many receptor combinations can be expressed by common or neighbouring neurons (section 1.3). The distribution of high affinity [^{3}H]-nicotine binding is very interesting, because it may reflect a single receptor isoform (α4ß2, see section 1.3) and there is a wealth of historical information in a variety of species (Wonnacott, 1987a), eg high affinity [^{3}H]-nicotine binding assays have been performed upon preparations of different human brain regions (Benwell and Balfour, 1985a), comparing smokers with non-smokers (Benwell *et al*, 1988).

The subcellular localization of nAChRs has been investigated by a variety of approaches:-

a) Immunostaining and mRNA hybridization techniques yield direct localization data:-

i) Some LGICs have been localized by histochemistry and immunoelectron microscopy of brain slices (Dechesne *et al*, 1990; Rogers *et al*, 1991a; Somogyi *et al*, 1989). In 1987 Swanson *et al*, used ^{125}I labelled monoclonal antibody (mAb) 270 to examine the distribution of the ß2 subunit in tissue slices at the light microscopy level, a virtually identical distribution described by Wada *et al*, (1989) using the more sensitive histochemical mRNA hybridization technique. These techniques were of insufficient to examine subcellular distribution, but sometimes the regional distribution of a receptor can be used to deduce its subcellular localization (Swanson *et al*, 1987). Recently, Hill *et al* (1993) performed immunoelectron microscopy studies upon tissue slices and obtained very similar results for the regional distribution of the ß2 subunit. Hill *et al* (1993) raised antibodies to the M3-M4 region of the ß2 subunit and then used two

immunological detection methods: peroxidase-linked nickel-enhanced [3,3',4,4'tetraaminobiphenyl]-tetrahydrochloride (3,3'-Diaminobenzidine, [DAB]) precipitation and silver-amplified immunogold detection; the latter technique is less sensitive, because immunogold labelling is less efficient, but localization can be more refined, because discrete particles are detected instead of a diffuse stain (see appendix A.3). At the subcellular level, both techniques revealed extensive cytoplasmic ß2 staining representing reserve, redundant or abortive expression; however no ß2 was detected in the Golgi and very little was found in the vicinity of synapses using both pre and postembedding procedures. This result may reflect detection problems or possibly non-synaptic localization (Sargent *et al*, 1989; Umbriaco *et al*, 1991). Hill *et al* (1993) did find some evidence for some postsynaptic ß2 expression in cortical neurophil dendrites, but no presynaptic terminal expression. These experiments are inconclusive with respect to α4ß2 localization, ß2 is expressed in other receptor subtypes and the results can be interpreted as showing pre- or post- or non-synaptic localization.

ii) The immunological staining of cultured neurons has been used to give nAChR localization information (Lippiello *et al*, 1991; Stollberg and Berg, 1987). These experiments have not resolved the issue of α4ß2 localization, in some cases (eg Lippiello *et al*, 1991) they produce a localization profile for a particular cell line, but even this information must be evaluated - the Langone/Bjercke antibodies used by Lippiello *et al* (1991) do recognize the α4ß2 subtype, but the recognition is not necessarily exclusive to this nAChR subtype.

iii) The distribution of nAChRs on subcellular particles has rarely been investigated by electron microscopy: Lentz and Chester (1977) reported the catalysis by peroxidase conjugated α-bungarotoxin of DAB deposition onto

postsynaptic densities associated with synaptosomes; Ratnam *et al* (1986a) demonstrated electron microscopy of immunogold stained postsynaptic vesicles formed by homogenization of neuromuscular junctions. Preliminary experiments, performed in our laboratory by Thorne (1989, person. comm.) using immunogold electron microscopy to examine the distribution of the $\alpha 4\beta 2$ subtype in subcellular particles, were compromised by a high non-specific signal.

b) Preliminary experiments, performed in our laboratory, seemed to show the immunoisolation of subcellular particles (synaptosomes) bearing the $\alpha 4\beta 2$ receptor; these synaptosomes were characterized for neurotransmitter content (Abstracts: Irons *et al*, 1988, 1989). The results indicated some $\alpha 4\beta 2$ localization to presynaptic terminals, however there was considerable experimental variation (Wonnacott person. comm.).

c) Ligand binding to subcellular fractions can localize receptor subtypes, if the ligand can distinguish the subtype. This approach has been used to examine $\alpha 4\beta 2$ localization by virtue of its high affinity [^{3}H]-nicotine binding. Early binding ligand studies to subcellular fractions indicated some $\alpha 4\beta 2$ were presynaptic (Benwell and Balfour, 1985b; Rapier *et al*, 1990; Yoshida and Imura, 1979). This result was recently reinforced by Thorne *et al* (1991) and Barnes *et al* (1992) who examined [^{3}H]-nicotine binding to subcellular fractions prepared on Percoll gradients which offer superior fractionation of synaptosomal material (Dunkley *et al*, 1987, 1988), however the results of [^{3}H]-nicotine binding studies to Percoll fractions may have been compromised by the specific binding of [^{3}H]-nicotine to Percoll (Wilkie and Wonnacott, 1991).

d) Functional assays can be used to detect and localize nAChRs. Nicotinic ligands have been shown to induce neurotransmitter release from synaptosomes prepared

by subcellular fractionation (Rapier *et al*, 1988, 1990; Rowell and Winkler, 1984; Wonnacott *et al*, 1989, 1990a). This is interpreted by the authors as confirming presynaptic nAChR localization and indicating that their role is to evoke or to modulate neurotransmitter release. It is possible that the nicotine acts on other channels to cause release, but the authors argued that the range of neurotransmitters, which are released in response to nicotinic agonists, and the accumulating pharmacological data characterizing release, strongly indicates that presynaptic α4ß2 nAChRs are responsible (Drasdo, 1993; Rapier *et al*, 1990; Sanderson, 1993; Wonnacott *et al*, 1989). Patch clamp analysis indicates functional α4ß2 nAChRs can be found postsynaptically on the soma (Mulle and Changeux, 1990) and dendrites (Aracava *et al*, 1987) of neurons.

e) Lesion experiments can be used to derive localization information by observing the resulting changes in receptor concentration in brain regions which contain different parts of the damaged neuron. Some lesion experiments show postsynaptic α4ß2 localization (Schwartz *et al*, 1984) others show presynaptic α4ß2 localization (Clarke *et al*, 1986; Clarke and Pert, 1985; Schwartz *et al*, 1984), this indicates that the receptor is found in different locations in different neurons, perhaps performing different functional roles (Papke, 1993).

f) A significant proportion of the nAChRs, which bind [^{3}H]-nicotine that are lost during Alzheimer's disease seem to be located presynaptically (Aubert *et al*, 1992; Giacobini, 1992; Kellar and Wonnacott, 1990)

It has been suggested that presynaptic α4ß2 represent as much as 30% of the total α4ß2 receptor population in some brain regions (Wonnacott, 1987a), this would indicate a significant role in synaptic modulation.

1.5 Synaptic modulation

The chemical synapse has multiple and dependent levels of regulatory control:-

a) many neurotransmitters are available (McGeer *et al*, 1987), each is recognized by a family of receptors consisting of several receptors subtypes, composed of distinct subunit combinations, with distinct pharmacological and perhaps functional profiles,

b) the neurotransmitter interacts with pre- or post synaptic receptors, which can be metabotropic or ionotropic, thus gradual and rapid changes can be made to either cell,

c) neuromodulators and hormones can regulate receptor responses (section 1.3),

d) combinations of neurotransmitters and neuromodulators can be released at the same synapse resulting in a composite response (O'Donohue *et al*, 1985),

e) the number of quanta released can be facilitated or depressed (Zucker, 1989), eg by changes in Ca^{2+} concentration,

f) the number of functional receptors can be altered, either by expression (permanent) or desensitization (temporary), (section 1.3). This is probably an important factor because the number of receptors can be low, some workers (Edwards *et al*, 1990; Larkman *et al*, 1991) report evidence from mammalian central nervous system (CNS) that the release of one neurotransmitter quantum (1 vesicle) can be sufficient to saturate all postsynaptic receptor sites (Stevens, 1993),

g) developmental changes in the synapse can result in changes in receptor expression (Goodman and Shatz, 1993) and even in the use of different neurotransmitters (Landis, 1980; Patterson and Nawa, 1993),

h) retrograde signals can result in the modulation of the presynaptic terminal by the post-synaptic cell, eg nitrous oxide is a retrograde messenger which causes long term potentiation (facilitation of release) in the presynaptic terminal (Jessel and Kandel, 1993; Stevens, 1993),

i) synaptic plasticity (Goodman and Shatz, 1993; Hall and Sanes, 1993) involves the precise organization of synaptic proteins by the bidirectional flow of information across the synapse (Hall and Sanes, 1993; Jessel and Kandel, 1993). This refinement and remodelling of synaptic connections continues throughout life (Edelman, 1993), reflecting the ability of the brain to adapt to experience (Patterson and Nawa, 1993).

Classically neuronal circuitry has been elucidated in simple neuronal systems such as *Aplysia* (Abrams *et al*, 1991) or, more recently, in model systems of low complexity where input can be quantitatively controlled and where the functional context of synaptic operations is clearly defined, eg in the visual and olfactory systems (review: DeVries and Baylor, 1993). It is a greater challenge to understand the interactions between neurons in other brain regions, which can involve 1000s or even 10,000s of synaptic connections (Edelman, 1993)! An appropriate approach might be functionally to characterize sub-populations of synaptic terminals.

1.6 Project aims

If the effects of nicotine upon the brain are to be elucidated, it is fundamentally important to define the functional role of nAChRs within complicated neural circuits. An essential aspect of nicotinic neurotransmission is receptor location; the precision of models for nicotinic responses will increase if receptor subtypes can be localized and their distribution correlated with neurotransmitters and other receptors. It was the aim of this project to develop the techniques used for preliminary experiments in our laboratory (section 1.4 aiii, b, c) to dissect the functional role of an nAChR isoform by examining its subcellular distribution, and by immunopurifying and characterizing subcellular particles which bore the receptor. The $\alpha 4\beta 2$ subtype was chosen, because of:-

i) its distinct pharmacological profile,

ii) the wealth of information, generally and within our group, about this isoform.

iii) the controversy surrounding the locations and roles of this isoform.

The project was divided into 3 experimental aims:-

a) To use physical separation techniques to reinforce the subcellular distribution of the $\alpha 4\beta 2$ isoform.

b) To use immunoaffinity techniques to immunopurify subcellular particles bearing the $\alpha 4\beta 2$ isoform.

c) To develop immunoelectron microscopy techniques to examine subcellular particles, from brain preparations and ultimately from immunopurified subcellular particles.

Chapter 2 Materials and equipment

2.1 Equipment

Centrifugation

High speed centrifugation was performed with a **Beckman L5-50B Ultracentrifuge** (Beckman Instruments), at 4°C using pre-cooled Beckman rotors: either a **SW50.1 swing-out** rotor holding 6 full **Beckman Ultraclear** centrifuge tubes (13x51mm, 5.2ml), or a **SW28 swing-out** rotor holding 6 full **Beckman Ultraclear** centrifuge tubes (25x89mm, 40ml), or, when these volumes were inappropriate, a **Ti50 fixed angle** rotor holding samples (0.5-6ml) in **Polycarbonate** tubes (Beckman).

Medium speed centrifugation was performed with a **Sorvall RC-5B** Refrigerated Superspeed centrifuge (Du Pont Instruments, Du Pont [UK] Ltd, Stevenage, Hertfordshire), at 4°C using pre-cooled fixed angle Sorvall rotors: either a **GSA** rotor (6x250ml **Sorval tubes**), or a **SA 600** rotor (12x50ml Sorvall tubes), or a **SM 24** rotor (outer ring - 16x14ml Sorvall tubes).

Low speed centrifugation of small volume samples was performed with a **MSE Micro Centaur microfuge and a Anderman Eppendorf Centrifuge (5415) varifuge**, if required, the entire centrifuge pre-cooled to 4°C for 4 hours before use. Samples were placed in **Eppendorf microtest** tubes (306/0308, BDH, Lutterworth, Leicestershire).

Colorimetry

When possible, samples were placed in 96 well microtitre plates (**Titretek multidish [flat bottom]** Nunc: Life Technologies Gibco Ltd, Paisley) and their optical density measured using a **Titretek multiscan MCC EFLAB** (ICN flow,

High Wycombe, Bucks). When a change of optical density was being measured or the relevent wavelength was not in the titretek repertoire, samples were placed in plastic cuvettes and measured with a **Pye Unicam SP6-450 spectrophotometer** (Pye Unicam Ltd, Cambridge) equipped with an electronic recorder.

Electron Microscopy

The resin was cured in **Gelatin capsules** (0.3ml A0487, Biorad: Fisons) or **Eppendorf microtest** tubes or a **Small flat embedding mould** (Biorad A0520) using a **Mini/696/Al incubator** (Genlab, Widnes, UK). Glass knives for sectioning were produced on an **LKB Knifemaker (LKB-Pharmacia 7801A)** and **LKB Trufs** (Biorad: Fisons A0584) were attached with wax. Sections were **cut** using a **Reichert OMU 3 Ultramicrotome** and mounted on **nickel** or **copper grids** (Biorad). Plastic film was cut using a **Diamond Scriber** (Biorad: Fisons A0564). Carbon coating was performed under vacuum in a **Speedyvac Coating Unit 12E6** (Edwards High Vacuum Ltd). Light microscopy was performed at x100 by oil immersion microscopy (**light microscope** - Carl Zeiss Jena Ltd, CZ Scientific Instruments Ltd). Transmission Electron microscopy was performed using a **1200 EX JEOL electron microscope** (JEOL, Watchmead, Herts). Photographs for analysis were made from developed film from the **1200** by projection onto photographic paper using a **DeVere 504 Projector** (Varicon) regulated by an **Electronic timer** (Rayco instruments Ltd).

Filtration

Filtration in ligand binding assays was performed using a **Brandel Cell Harvester M 24R** (Brandel: Semat, Herts) under a vacuum, of approximately 15 mm Hg,

provided by an **Edwards 5 two stage pump** (Severn Science Ltd, Thornberry, Bristol). All other filtration was performed using a **Buchner** filter (Fisons).

Mixing and tissue dispersion

Tissues were homogenized using a **glass-Teflon homogeniser**, total clearance 0.31mm, attached to a **varicontrol motor**, (Citenco F.H.P. motors).

Preparations were mixed using: a **Whirlimixer** (Fisons, Loughborough) when speed was important and tissue damage was not important; a **Pasteur pipette or Gilson pipette** for more gentle, less rapid mixing; or a **homogeniser** (as above) when specimen disruption was the primary concern.

Preparations were shaken using: a **Luckham R100 Rotatest Shaker** (Luckham Ltd: Denley Instruments Ltd, Billingshurst Sussex) for routine agitation; an empty water bath was used for gentle horizontal shaking of nitrocellulose sheets, etc; a home-made **end-over-end** shaker was used for very gentle agitation, eg for the attachment of material to beads or for the preparation of material for electron microscopy.

Magnetic separation

The isolation of magnetic beads was achieved using a **Dynal MPC-1 magnetic particle concentrator** (Dynal, New Ferry, Wirral).

Polyacrylamide gel electrophoresis and western transfer

The Phast system (Pharmacia [GB] Ltd, Hounslow, Middlesex) was used to perform PAGE, transfer onto nitrocellulose was achieved using the add-on

transfer unit. **The Mighty Small II Minigel system** (SE 250 Hoefer Scientific Instruments) was also used to perform PAGE, transfer onto nitrocellulose was achieved using the **TE series Transphor electrophoresis unit** (Hoefer).

Preparation of solutions

a) The Osmolarity of solutions was measured using a **freezing point osmometer** (Camlab Ltd, Roebling).

b) Radioligands and (-) nicotine were dispensed using a **Drummond 10μl micro-dispenser**.

c) Percoll gradients were layered using a **Gilson Minipuls 2 Peristaltic pump** (Gilson: Anachem, Luton) with a fine bore **25Gx15/16 inch Sabre** needle (Gillette UK Ltd, Isleworth, Middlesex, UK)

Radioactivity detection

a) A **1600 TR, Tri-Carb Liquid Scintillation analyzer** (Packard Instrument Company: Canberra) was used to measure Tritium.

b) A **1277 Gammamaster automatic gamma counter** (Wallac: LKB-Pharmacia, Milton Keynes) was used to measure ^{125}I.

c) ^{125}I labelled material was detected by **autoradiography** using Tritium sensitive film (see photography) which was developed using a **Amersham Hyperprocessor developing unit**.

2.2 Materials

Common buffers

Electrophoresis sample buffer (prepared fresh) contains: 0.1M Tris, 0.1M bicine, 10% w/v sucrose, 2% w/v SDS, 5% v/v ß-mercaptoethanol, 0.05% w/v Bromophenol blue.

PBS - 10mM Phosphate Buffered Saline (prepared fresh) contains: 8mM potassium dihydrogen phosphate (KH_2PO_4), 2mM dipotassium hydrogen phosphate ($K_2HPO_4.3H_2O$), 150mM sodium chloride (NaCl). pH adjusted to 7.4

Tris/HEPES (stored at -20°C, or < 5days at 4°C) contains: 118mM sodium chloride (NaCl), 4.8mM potassium chloride (KCl), 2.5mM calcium chloride ($CaCl_2$), 20mM N-[2-Hydroxyethyl] piperazine-N-[2-ethane sulfonic acid] (HEPES), 200mM Tris, 0.1mM Phenylmethylsulfonyl fluoride (PMSF), 0.01% sodium azide. pH adjusted to 7.4

Electron microscopy reagents

Epoxyresin (Epoxyresin Kit TK1, medium hardness Araldite CY 212 resin from TAAB Laboratories Eq Ltd.) cured by heat cure (48 h 60°C). **LR White resin** (LR White resin medium hardness L012, TAAB Laboratories Eq Ltd) cured by cold cure (24h, -15°C) using the accelerator or by heat cure (24h, 55°C).

Colloidal gold labelled antibodies and Avidin - see immunological reagents.

Reynolds lead citrate was prepared as follows. Lead citrate (2.66g) was mixed with sodium citrate (3.52g) in 60ml water and incubated with gentle agitation at room temperature for 30 min. The pH of the staining solution was raised to pH

12.0 by the addition of 1M NaOH (8ml) and water (to a final volume of 100ml). The mixture may be kept at 4°C for 6 months but should be filtered before use.

Immunological reagents and stains

[^{125}I]-Avidin see radiochemicals

Lindstrom antibodies and **Rogers** antibodies refer to Table 2.1. Stored at -20°C in 10μl aliquots.

Colloidal gold probes, stored at 4°C:-

a) **Auroprobe TM EM Goat-(anti-Rat) G10**: anti-rat IgG probes conjugated to 10nm diameter gold particles. (Amersham)

b) **Goat Anti-rat IgG (whole molecule) gold conjugate 5nm** (Sigma G2397)

c) **Streptavidin Albumin conjugated to 10 nm Colloidal gold** labeled from *Streptomyces avidinii* (Sigma S4275)

Peroxidase linked probes, stored at 4°C:-

a) **Extravidin peroxidase conjugate** (Sigma Chemical Co Ltd, Poole, Dorset, E-2511)

b) **Goat-(anti-Rat IgG [whole molecule]) peroxidase conjugate** (Sigma A-9037)

c) **Rabbit-(anti-Rat IgG [whole molecule]) peroxidase conjugate** (Sigma A-5795)

d) **Vectastain Elite ABC Kit, Standard** (Vector PK 6100)

Linker proteins, stored at 4°C:-

a) **Goat-(Anti-rat IgG [whole molecule])** (Sigma R5005)

b) **Protein A** Extracellular from *Staphylococcus aureus* (Sigma P6031)

c) **Protein G** from Group C *Streptococcus* sp. (Sigma P9659)

Protein blocking, stored at 4°C:-

a) **Goat serum** (Sigma)

Table 2.1 The antibodies used for the project

Antibodies donated by J. Lindstrom. Raised in rats against detergent solubilized rat nAChRs. Henceforth collectively refered to as Lindstrom mAbs:

mAb	conc mg/ml	Titre µM	vs	Immunogen (brain nAChR of)	Class
270	1.5	1.6	ß2	chick	IgG2a
290	40	26.9	ß2	rat	IgG1
297	40	0.3	ß2	rat	not IgM
299	115	0.3	α4	rat	not IgM

Polyclonal sera donated by S.Rogers. Raised in rabbits against rat nAChR fusion proteins. Henceforth collectively refered to as Rogers sera:

antiserum	5009	4842
vs	α4	ß2
immunocytochemistry	1:500 - 1:1000	1:1500 - 1:3000
immunohistochemistry	---	1:2000 - 1:3000
immunoprecipitation	1:800	1:500 - 1:1000

b) **Vectastain Avidin D/biotin blocking Kit** (Vector laboratories, Bretton, Peterborough, England SP 2001)

c) **Marvel dried skimmed milk** (Chivers and Sons Ltd, Coolock, Dublin), prepared fresh.

Stains:-

a) **5-aminosalicylic acid** (5-Amino-2-hydroxybenzoic acid, [5AS] (Sigma A6178)

b) **3,3'-Diaminobenzidine** ([3,3',4,4'tetraaminobiphenyl]-tetrahydrochloride, [DAB]) Sigma D5637

c) **paranitrophenyl phosphate** (p-nitrophenyl phosphate, disodium salt, pnpp) (Sigma 104-0)

d) **PAGE blue 83** (BDH 44246)

e) **Ponceau S Red** (Sigma P7170)

Photographic materials

The 1200 EX uses **Black and white negative film, Kodak Plux-Xpan**, (5-PXP 200, Eastman Kodak Co. Rochester, N.Y., USA), which was developed using **Ilford Ilfofix fixer and Ilford Perceptol developer** (Ilford Ltd: CIBA-GEIGY). **Ilford RC De luxe Multigrade II** (MG.1M 24x30.5cm, Ilford) photographic paper was developed with **Ilford Multigrade paper developer** (Ilford) and fixed with **Ilford Hypan fixer** (Ilford).

Hyperfilm [^{3}H] High Performance autoradiography film (RPN 535, Amersham)

Preparation of discontinuous gradients

Gradients were made using freshly prepared solutions using double distilled water and equipment which had been washed thoroughly to remove all trace of

detergent. All gradients were prepared using chilled solutions (4°C), where the pH had been adjusted to 7.4. In all cases the sample was initially suspended in 0.32M sucrose (pH 7.4).

0.8/1.2M sucrose gradients were freshly prepared in either 5.2ml or 40ml Beckman Ultra-Clear centrifuge tubes, the volumes used were appropriate to fill the tubes. A pasteur pipette was used to layer gradients comprising of 1 volume of sample, on 2 volumes of 0.8M sucrose, on 2 volumes of 1.2M sucrose.

0.8/1.0/1.2M sucrose gradients were freshly prepared in 5.2ml Beckman Ultra-Clear centrifuge tubes, the volumes used were appropriate to fill the tube. All 4 sucrose solutions, including the sample solution, contained 50μM calcium chloride $CaCl_2.2H_2O$. A pasteur pipette was used to layer gradients comprising of 1 volume of sample, on 1 volume of 0.8M sucrose, on 1 volume of 1.0M sucrose, on 1 volume of 1.2M sucrose solution.

Percoll gradients were prepared in 14ml Sorvall centrifuge tube and comprised of equal (2ml) volumes of:- sample (0%), on 3%, on 10%, on 15%, on 23% v/v Percoll suspensions in 0.32M sucrose. Percoll was filtered using a Millipore AP 15 prefilter to remove aggregates. Percoll suspensions were prepared in 0.32M sucrose and the pH adjusted to 7.4. All suspensions were layered using a Gilson Peristaltic pump (running speed 1ml/min) set up with a Sabre fine bore needle. Gradients were allowed to settle for at least one hour before sample application, they could be stored at 4°C for up to 24 h.

Radiochemicals

[^{125}I]-Bolton Hunter labelled Avidin (CG92520, New England Nuclear [NEN] research products, Du Pont [UK]). Thawed, diluted (10% v/v) with Phosphate

Buffered Saline (PBS) containing (0.1% w/v) Bovine Serum Albumin [BSA] and stored at -20°C in 200μl aliquots for storage. Specific activity 157 μCi/μg, concentration 92.4 μCi/ml, $T_{½}$ is 60 days. The [^{125}I]-Avidin was stable and usable for 4 weeks, but for maximal activity it was used within 10 days of the preparation (89% original activity).

[^{3}H]-cytisine:- cytisine HCl [3,5-^{3}H(N) cytisine] approximately 30 (15-40)Ci/mmol, 30μM in 90% ethanol (Net-1054, NEN), stored at -20°C.

[^{3}H]-nicotine:- (-)-N-methyl-^{3}H-nicotine approximately 75 (60-87)Ci/mmol, 15μM in 90% ethanol (Net-827, NEN), stored at -20°C in 100μl aliquots in a ten fold molar excess of ß-mercaptoethanol (Romm *et al*, 1990)

Solid supports:- filters, gels and plates

Supports, stored at 4°C:-

a) Agarose-Avidin D (Vector A2010)

b) cyanogen bromide activated Sepharose 6MB (Sigma C9267)

c) tosylactivated Dynabeads M-450 (Dynal (UK) Ltd, New Ferry, Wirral, UK 14003/14004)

d) Dynabeads M280 Streptavidin (Dynal 112.05/112.06)

e) Dynabeads M450 Sheep anti-rat IgG (11007/11008)

f) Pansorbin cells *Staphylococcus aureus* based immunoadsorbents (Calbiochem-Novabiochem [UK] Ltd, Highfields Science Park, Nottingham, UK.)

Polyacrylamide electrophoresis (Phast system), stored at 4°C:-

a) Phast Gel: SDS Buffer Strips, (Pharmacia, 17-0516-01)

b) Phast Gel: Gradient Gel 10-15%, (Pharmacia, 17-0540-01)

c) Phast Gel: Sample Applicator 8/1, (Pharmacia, 18-1618-01)

Polyacrylamide electrophoresis (Mighty Small II):-

a) 10% Polyacrylamide gel (running gel). 0.1M Tris (hydroxymethyl) amino methane [Tris], 0.1M N,N-bis(2 hydroxyethyl) glycine [Bicine], 10% w/v acrylamide, 0.27% w/v bis acrylamide, 0.1% w/v sodium dodecyl sulphate [SDS], 0.1% v/v N,N,N',N'Tetramethyl ethylenediamine [TEMED], after mixing add 0.09% w/v ammonium persulphate. A few drops of 50 % v/v ethanol was layered onto the surface of the gel after pouring to improve gel shape. Gels may be stored before use in a humid atmosphere at 4°C for up to 5 days.

b) 2.5% Polyacrylamide gel (stacking gel). 0.1M Tris, 0.1M bicine, 2.5% w/v acrylamide, 0.067% w/v bis acrylamide, 0.1% w/v SDS, 0.2% v/v TEMED, after mixing add 0.03% w/v ammonium persulphate. Stacking gels were always freshly prepared.

Filters:-

a) Gelman GFA/E Glassfibre filters

b) Nitrocellulose filter BA 85 Membrane Filters (0.45μm) (Schleicher and Schuell)

c) Millipore AP 15 filter (Millipore UK Ltd, Watford, Herts)

Miscellaneous

Optiphase "safe"scintillant (LKB Scintillation Products). "Super polyethylene vial" standard (20ml) scintillation vial (Packard). Lp4 tubes (Luckham). Glycopeptidase F (EC 3.2.2.18)from *Flavobacterium meningosepticum* (Sigma G9769), stored at 4°C. Collagenase from Boehringer Mannheim (103 586), freshly prepared before use. All other chemicals brought from Sigma, Fisons or BDH as appropriate.

Chapter 3 The characterization of subcellular fractions by ligand binding and by immunostaining techniques other than immunoelectron microscopy

3.1 Introduction

This section describes experiments which were performed to reinforce the findings of parallel attempts at immunoelectron microscopy (section 4) and immunoisolation (section 5) of nerve terminals bearing the nAChRs. These experiments are divided into two approaches:-

a) The subcellular localization of nAChRs was investigated by examining subcellular fractions using ligand binding and a combined approach of ligand binding and receptor immunoprecipitation.

b) The immunological recognition of the α4ß2 receptor subtype was investigated by a variety of biochemical techniques.

3.1.1 Subcellular localization

The α4ß2 nAChR subtype is mainly or wholly responsible for high affinity [^{3}H]-nicotine or [^{3}H]-cytisine binding (section 1.3). Various investigations have demonstrated that part of the α4ß2 population is located at presynaptic terminals (section 1.4). Previous [^{3}H]-nicotine binding studies to subcellular fractions have shown that the α4ß2 isoform is enriched in synaptosomal fractions (see sections 3.2.2 and 3.2.4).

The measurement of [^{3}H]-nicotine or [^{3}H]-cytisine binding can be problematic (Romano and Goldstein 1980). It is not within the scope of this thesis to present a detailed description of the problems and the best approaches to this assay, because a good system had been established in the laboratory before this work

began (MacAllan *et al*, 1988), but the limited characterization which was performed is described in section 3.3.1. The [^{3}H]-cytisine binding experiments described in sections 3.3.3 and 3.3.4 were designed to develop methods to measure $\alpha 4\beta 2$ concentrations in S1-Percoll fractions, as described by Thorne *et al* (1991) and by Barnes *et al* (1992), whilst circumventing the binding of [^{3}H]-nicotine to Percoll (Wonnacott and Wilkie, 1991), which might compromise results. The use of Western blotting to localize the glutamate receptor GluR1 to the post synaptic density preparation (Rogers *et al*, 1991a) initiated two lines of research. Section 3.1.2 describes Western blotting attempts. Section 3.3.2 describes an attempt to use [^{3}H]-nicotine binding experiments to determine the proportion of $\alpha 4\beta 2$ which can be measured at synaptic junctional complexes (SJC). Note sections 3.3.2and 3.3.4describe preliminary localization experiments which were performed at the end of the project. In both cases some improvements were possible, which might increase the accuracy of results (sections 3.4.2 and 3.4.3), but no change in the qualitative picture of receptor localization was expected.

3.1.2 Immunological recognition

Whiting and Lindstrom (1987, 1988) reported immunoblot experiments with the Lindstrom mAbs used in this project:- immunopurified receptors were separated by PAGE, transferred by Western transfer onto nitrocellulose and immunostained using the mAbs detected by ^{125}I-labelled goat (anti-rat IgG) antibodies. The experiments discussed in section 3.3.5.2 were performed to determine if the sensitivity of the Western blot system was sufficient to allow the analysis of preparations without immunopurification. Rogers *et al* (1991a) demonstrated that such an analysis can provide a semi-quantitative procedure to examine the

localization of receptors. Dot blot controls were performed to confirm the detection of nitrocellulose-bound primary probes by labelled secondary probes. The experiments discussed in section 3.3.6 were partly performed with the same objective, ie to determine if ELISA was sensitive enough to allow the analysis of preparations without immunopurification. Neither Western blot nor ELISA were sensitive enough to be used for receptor analysis without immunopurification.

Immunoelectron microscopy is a labour intensive procedure and so controls are often performed to ensure that immunostaining is possible before an extensive study is performed. One of the primary aims of the project was to attempt to immunostain synaptosomes bearing the nAChR. The normal control prior to immunoelectron microscopy is to immunostain a preparation for light microscopy (eg Hill *et al*, 1993), however all component parts of a synaptosome preparation are too small to be visible using light microscopy, and so any specific immunostaining could not be identified or quantified. Light microscopy of an immunostained tissue block (Swanson *et al*, 1987) was a poor control for immunostaining synaptosomes, because the immunostaining conditions are different. Sections 3.3.6 and 3.3.7 describe the development of immunostaining controls which would allow the quantification of specific nAChR recognition. The development of immunostaining controls was performed at the same time as the preliminary immunoelectron microscopy experiments described in section 4, they reinforce the results described in sections 4 and 5, which indicate that nAChR recognition by mAbs was unexpectedly compromised under the experimental conditions used.

3.2 Methods

The methods described in this chapter are divided into tissue preparation (sections 3.2.1 to 3.2.4) and detection (section 3.2.5 to 3.2.10) procedures.

Tissue preparation procedures

Unless otherwise stated, throughout all procedures described in section 3.2.1 to 3.2.4: all tissue preparation was performed at 4°C using chilled solutions and equipment; all vessels were washed carefully in distilled water to remove all trace of detergents; all pellet resuspension was performed by homogenization.

3.2.1 Homogenization and summary of tissue preparations

Male Sprague-Dawley rats (age 4-6 wk, mass 200-250g) were obtained from a breeding colony maintained at the University of Bath. They were killed by cervical dislocation and the whole brain minus cerebellum (mass approx. 1.2g) rapidly removed. Brain dissection to obtain cortex (mass approx 0.75g) was performed on ice, whilst soaking in homogenizing buffer containing sucrose (0.32M), EDTA [Diaminoethane tetra-acetic acid disodium salt] (5mM), PMSF (0.1mM), pH7.4. To minimize postmortem changes, brain tissue (cortex or whole brain minus cerebellum) was rapidly placed into homogenizing buffer (10% w/v) and homogenized at the earliest opportunity, using 2x6 up/down strokes of a homogenizer at 200 rpm. Figure 3.1 outlines all the tissue preparations made from the homogenate by differential centrifugation.

Figure 3.1 The tissue preparations made from homogenate by differential centrifugation

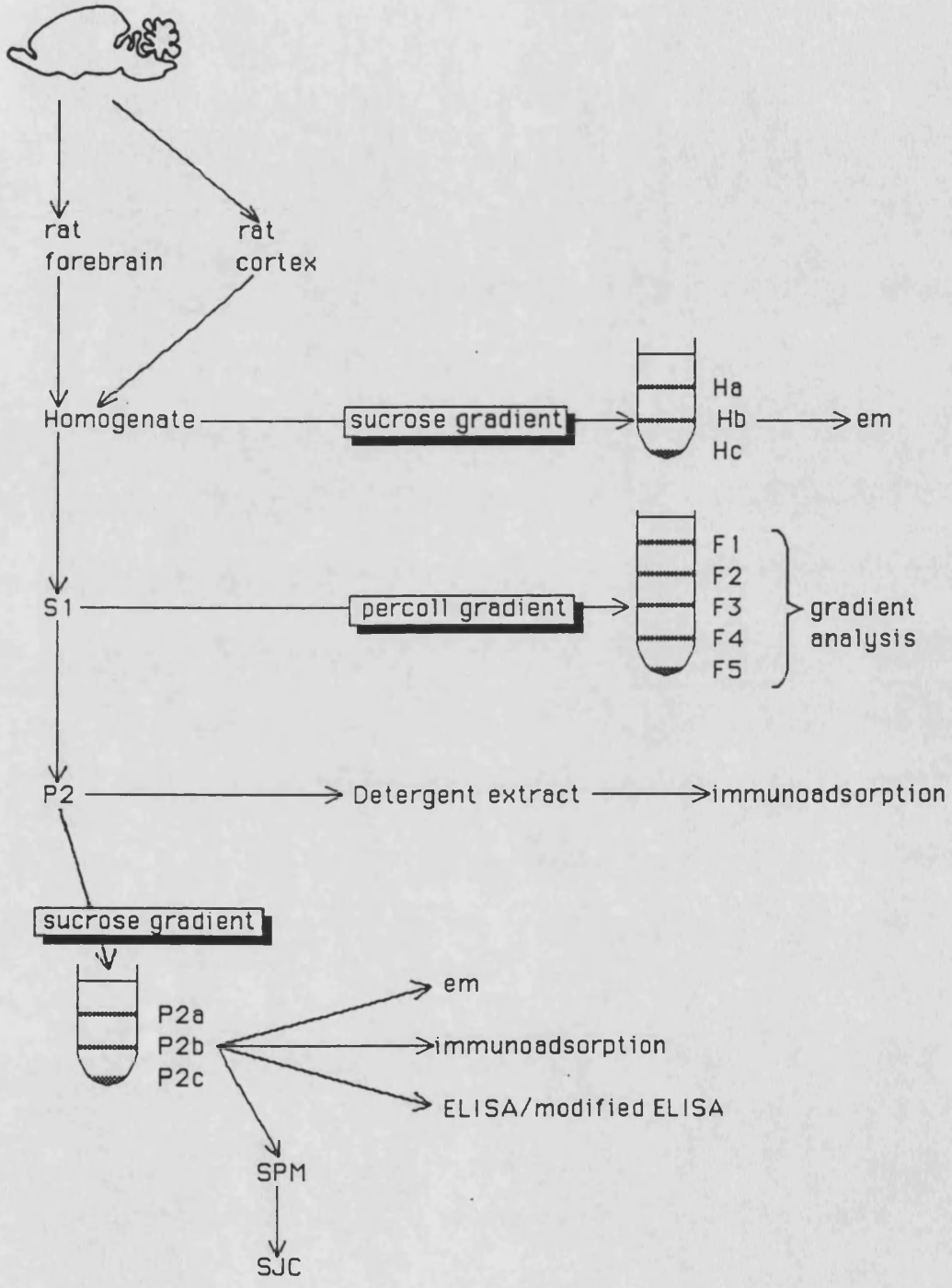


Figure 3.1 summarizes the tissue preparations and the uses to which they were put. For greater details of preparative procedures see figures 3.2, 3.3, 3.4 and 5.1.

3.2.2 Synaptosome isolation by subcellular fractionation using sucrose gradients

Brain homogenate was fractionated using a 2-step sucrose density gradient (figure 3.2), essentially as described by Rapier *et al* (1988). Briefly, the homogenate was layered onto a 0.8/1.2M sucrose gradient (section 2.2) and after centrifugation (100,000g, 60 min, Beckman, swing out rotor), the fractions were collected from sucrose interfaces using a Pasteur pipette. The synaptosome preparation from the 0.8/ 1.2 interface (Hb) contains a considerably higher concentration of high affinity [^{3}H]-nicotine binding sites than the other 2 fractions (Rapier *et al*, 1990).

Alternatively, differential centrifugation of the brain homogenate yields a P2 plasma membrane preparation (Gray and Whittaker, 1962; Rapier *et al*, 1990), as follows (figure 3.2). Centrifugation (Sorval) of the homogenate at 1,000g, for 10 min, yielded a supernatant fraction (S1) and a pellet (P1). This pellet was resuspended in fresh homogenizing buffer (5ml/g original wet weight) and the supernatant fraction from centrifugation of the resuspended P1 (1,000g, 10 min) was pooled with the S1. Centrifugation (17,000g, 30 min) of pooled S1 yielded a supernatant fraction (S2) and a pellet (P2). This pellet was resuspended in fresh homogenizing buffer (2.5ml/g original wet weight). The P2 plasma membrane preparation could then be fractionated (Beckman, swing-out rotor, 100,000g, 60 min) using a 2-step (0.8/1.2M) sucrose density gradient (Gray and Whittaker, 1962) using the same procedure as described above for Hb (figure 3.2). The P2b preparation consists of approximately 50% v/v synaptosomes (Whittaker, 1969) and contains a considerably higher concentration of high affinity [^{3}H]-nicotine binding sites than the other 2 fractions (Yoshida and Imura, 1979).

Figure 3.2 The isolation of synaptosome preparations using sucrose gradients

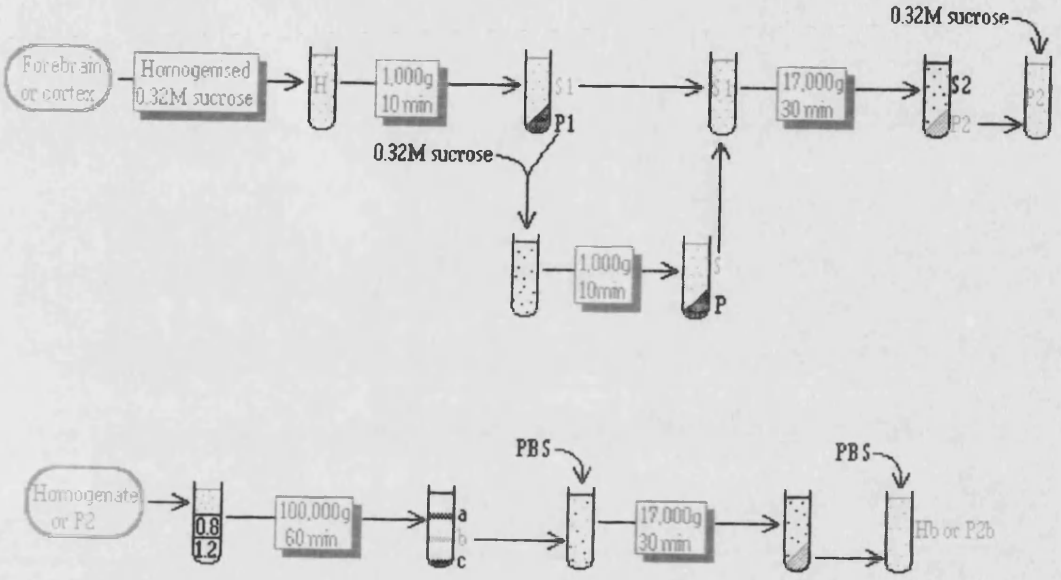


Figure 3.2 summarizes the production of the Hb (Rapier *et al*, 1990) and P2b (Gray and Whittaker, 1962) synaptosome preparations using sucrose density gradients.

3.2.3 The preparation of synaptic junctional complexes

The production of synaptic junctional complexes from the P2b synaptosome preparation (figure 3.3) was based on the procedures described by Cotman and Taylor (1972), and by Rao and Steward (1991). The P2b preparation was gently shaken with four volumes of 10mM PBS, then after centrifugation (17,000g, 30 min, Sorval) the synaptosomes in the P2b pellet were lysed. The pellet was twice resuspended in 0.1 mM HEPES (1% w/v) and left for 15 min before centrifugation (23,000g, 15 min). The pellet was washed by centrifugation with 0.16M sucrose, 50μM calcium chloride ($CaCl_2$), ie the pellet was resuspended (1% w/v, 0.16M sucrose/$CaCl_2$) and immediately precipitated by centrifugation (23,000g, 15 min). The pellet was resuspended (1% w/v) in a warmed sodium phosphate buffer (30°C, 40mM, pH 7.6) which contained 0.5 mM iodonitrotetrazolium violet and 20 mM succinate and incubated at 30°C for 20 min. This treatment reduces the density of mitochondria so that they do not precipitate during centrifugation (10,000g, 7 min). The pellet was washed twice by centrifugation (10,000g, 7 min) with 0.16 M sucrose, 50μM $CaCl_2$ (1% w/v) and resuspended (approx 4ml/g rat brain) in 0.32M sucrose, 50μM $CaCl_2$. This mixture was layered onto a 3-step sucrose density gradient (0.8/1.0/1.2M). After centrifugation (75,500g, 90 min, Beckman, swing-out rotor) the material from the 1.0/1.2 interface was collected. This fraction represents the synaptic plasma membranes (SPM), it was mixed with three volumes of 50μM $CaCl_2$. The pellet from centrifugation (10,000g, 10 min) of this mixture was resuspended using a pasteur pipette in 2mM bicine, 2mM EDTA, 0.4% Triton X-100 (1.5ml/g rat brain) and left for 10 minutes at room temperature. The mixture (1 volume) was layered onto 1.0M sucrose, 50μM $CaCl_2$ (4 volumes) for centrifugation (68,000g,

Figure 3.3 The isolation of synaptic junctional complexes from P2b preparations

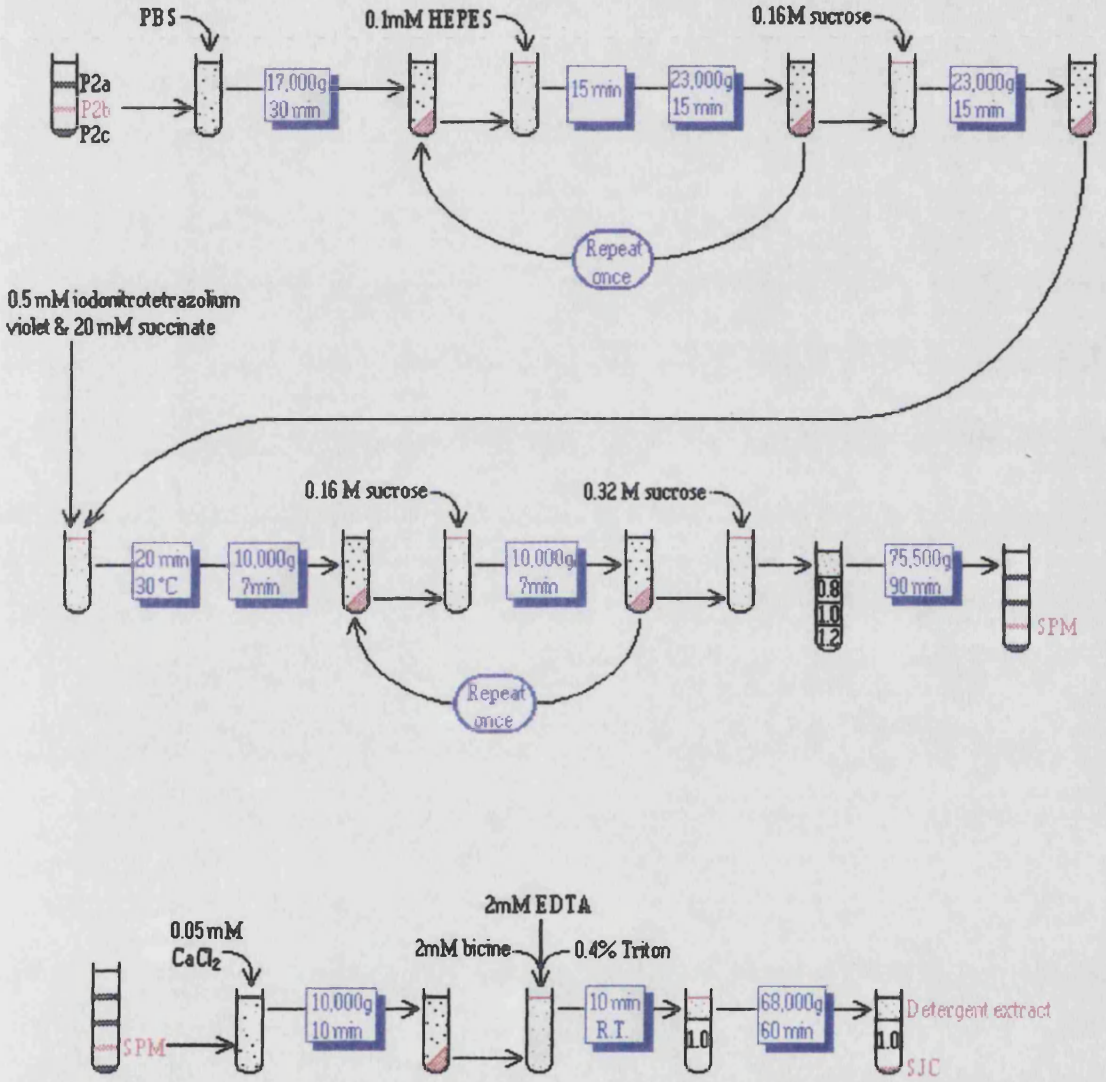


Figure 3.3 summarizes the production of synaptic junctional complexes (SJCs) from P2b preparations, based on the protocol described by Cotman and Taylor (1972), and by Rao and Steward (1991).

60 min, Beckman, swing-out rotor). The pellet consisted of synaptic junctional complexes (SJC), and the extract floating on the sucrose represented the non-SJC component of SPM.

3.2.4 Subcellular fractionation using Percoll gradients

An alternative fractionation technique involves only 2 rapid medium-speed centrifugation steps (figure 3.4): the S1 Percoll procedure (Dunkley *et al*, 1987, 1988; Harrison *et al*, 1988) is a non-equilibrium method which separates particles on the basis of size and density. Rat neural tissue (cortex or whole brain minus cerebellum) was homogenized in fresh 0.32M sucrose (pH 7.4). Homogenate centrifugation (Sorval, 1,000g, 10 min) yielded the supernatant S1 which was layered onto chilled discontinuous 4-step Percoll gradients (section 2.2). Centrifugation (5 min, 32,500g) yielded 4 interfacial fractions and a pellet. All 5 fractions contain synaptosomes, but fractions F1, F2 and F5 contain synaptosomes of low viability (Harrison *et al*, 1988) and considerable non-synaptosomal material (Dunkley *et al*, 1988), whilst the F4 fraction is especially rich in synaptosomes (56% v/v, very strict criteria, Dunkley *et al*, 1988) of high viability (Harrison *et al*, 1988). The concentration of high affinity [^{3}H]-nicotine binding sites increases down the gradient, ie increases from fractions F1 to F5 (Barnes *et al*, 1992; Thorne *et al*, 1991).

Detergent extracts of each Percoll fraction (figure 3.4) were made before quantification of high affinity nicotine binding sites; the supernatants from detergent extraction were assayed either by immunoprecipitation (section 3.2.10) or by filtration (section 3.2.6).Before detergent extraction fractions were washed

Figure 3.4 The isolation of synaptosome preparations using Percoll gradients

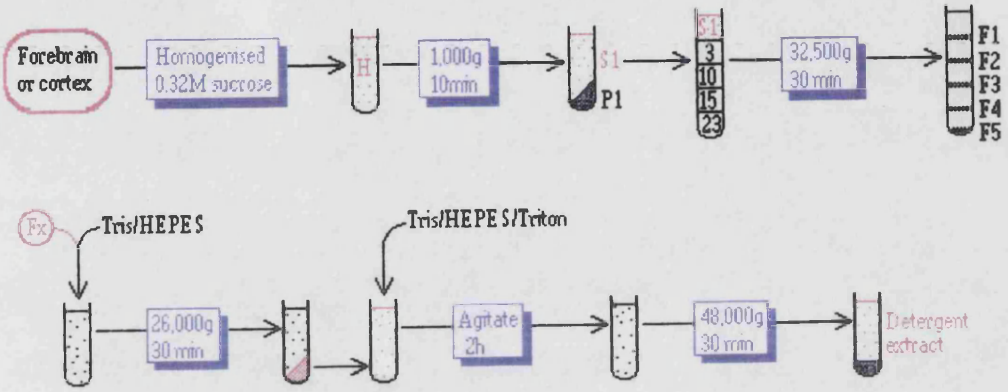


Figure 3.4 summarizes the procedure for the subcellular fraction of the S1 preparation using Percoll gradients (Dunkley *et al*, 1987, 1988; Harrison *et al*, 1988).

once, by centrifugation, with 20 volumes of Tris/HEPES buffer (26,000g, 30min). The pellets were resuspended using a pasteur pipette in 1 volume of Tris/HEPES containing 0.5% v/v Triton X-100, then incubated for 2 h, under gentle agitation. The supernatant from centrifugation (Beckman, 48,000g, 30 min, fixed angle rotor) was collected.

Detection procedures

3.2.5 Protein estimation

Two methods were used to measure protein concentration.

3.2.5.1 The Lowry method

Protein estimation was performed essentially as described by Lowry *et al* (1951) using BSA as a protein standard. A constant and minimal buffer concentration was maintained in all tubes including standards and blanks, to avoid interference effects from buffer components. All samples were measured, in duplicate, at two dilutions, in the protein concentration range 0-300 μg/ml.

Fresh copper complex solution (1ml) containing: NaOH (0.1M), sodium carbonate (2% w/v Na_2CO_3), copper sulphate (0.01% w/v $CuSO_4.5H_2O$), sodium tartrate (0.02% w/v $[CHOHCOONa]_2.2H_2O$) was vortex mixed with diluted samples (200μl) in LP4 tubes. After a 10 min incubation at room temperature, each sample was vortex mixed with Folins-Ciocalteau reagent (100μl). After a further 40 min incubation, aliquots (2x200μl) of each sample were transferred to microtitre plates for optical density measurement, at 690nm, using a titretek

multiscan reader.

The procedure for the estimation of protein in samples which contained detergents was identical, except the copper complex solution also contained 1% w/v SDS (Markwell *et al*, 1978).

3.2.5.2 Protein approximation by PAGE analysis of dilution series

A crude approximation of protein concentration was made by analyzing several dilution series of a sample using PAGE (section 3.3.5.1).This technique is not affected by the composition of the sample buffer, or the presence of other proteins unless they are composed of polypeptides of the same molecular weight. It was used when a Lowry estimation was not possible, eg to measure antibodies in an unknown buffer, which contained stabilizing proteins. The vanishing point where a particular polypeptide was at a concentration which was just undetectable was assumed to be constant for that polypeptide. A series of serial dilutions were used to "home in" on the vanishing point dilutions of polypeptide components of a protein. Pure protein preparations (no stabilizing proteins) of known protein concentration (estimated by Lowry) were used as standards.

3.2.6 Ligand binding assays

The detection of nAChRs using the filtration assay was based on the procedure first described by Romano and Goldstein (1980). The assay was performed, essentially as described previously by our laboratory (MacAllan *et al*, 1988), except samples were suspended in Tris/HEPES buffer (see section 2.2) to reduce non-specific binding (Romm *et al*, 1990). When binding assays were performed on

detergent solubilized receptors care was taken to use Tris/HEPES buffer containing 0.25% v/v Triton X-100, unless otherwise stated. Total and non-specific high affinity binding were measured in triplicate.

Tritiated nicotine binding proceeded as follows:-

Samples (6x0.5 ml, 0.5-1.0 mg protein/ml) were placed in LP4 tubes. Water (10μl) was added to measure total binding, or (-) nicotine free base (10μl, final assay concentration 1mM) was added to measure non-specific binding. All tubes were vortex mixed. The [^{3}H]-nicotine (10μl, final assay concentration 20nM) was added to each tube and all tubes were vortex mixed. The tubes covered and incubated for 30 min at room temperature, then for 2 h at 4°C. Samples were rapidly filtered under vacuum (4°C) using a Brandel cell harvester (Lippiello and Fernandes, 1986), through a double thickness of GFA/E filters which had been pre-soaked (18hr, 4°C) in PBS containing polyethleneimine (PEI: 0.3% v/v) and azide (0.05% w/v), and washed with PBS/azide (2x4ml, 4°C). The PEI exposure had 2 effects:- it reduces the non-specific binding (Lippiello and Fernandes, 1988; Schwartz *et al*, 1982) of [^{3}H]-nicotine and radiolabelled impurities (Romm *et al*, 1990) to the glass fibre filters (Romano and Goldstein, 1980) and it allows the adsorption of detergent solubilized nAChRs by ion exchange (Bruns *et al*, 1983; Lunt, 1987). The total filtration and washing time was less than 30 seconds. Filters were placed in scintillation vials. Scintillation fluid (5ml) was added and, after 4h, the vials were vortex mixed. The level of Tritium was measured with controls in the 1600 TR.

Tritiated cytisine binding was performed in the same way as nicotine binding

(Pabreza *et al*, 1991), except:- [^{3}H]-cytisine (10μl, final assay concentration 2.5nM) was added instead of [^{3}H]-nicotine; tubes were incubated for 2h at room temperature.

3.2.7 Polyacrylamide gel electrophoresis

3.2.7.1 Electrophoresis procedures

PAGE was performed as described by Laemmli (1970). Two electrophoresis systems were used:-

a) Electrophoresis using the Phast system was mainly used for protein estimation (section 3.3.5.1).As recommended, samples were incubated (10min, 100°C, 50% v/v) in sample buffer (section 2.2), before electrophoresis (30 min, 10mA/gel) on precast running gels (10-15% gradient) with buffer strips. Western transfer (Renart *et al*, 1979) onto nitrocellulose was achieved using the transfer attachment (15 min, 20 mA/gel).

b) The Minigel system was used for immunoelectrophoresis experiments, as described by Peers *et al*, 1993. Samples were incubated in sample buffer, as above, and then the reduced SDS-bound samples were applied (20μl) to a 1.5mm 10% running gel (section 2.2), with 2.5% stacking gel (section 2.2). Electrophoresis using a running buffer containing Tris (0.1M), bicine (0.1M), SDS (0.1% w/v) proceeded at 10 mA/gel until samples had passed through the 2.5%-10% gel interface (approx 10 min) and then with a current of 35mA/gel for a further 1h (60 min, 35 mA per gel). Western transfer onto nitrocellulose was performed (constant 80V, 1h) in transfer buffer containing Tris (2.5mM), Glycine (19.2mM), methanol (20% v/v).

3.2.7.2 Protein visualization after PAGE

All incubations described in this section were performed at room temperature, under gentle agitation.

Protein was visualized in gels, as directed, by staining (2 h) with PAGE blue 83 (0.2% w/v in 50% v/v methanol, 10% v/v acetic acid) and then destaining (24 h) with 20% v/v methanol, 10% v/v acetic acid. Stained gels could be kept for months in water, or dried for storage.

Protein was visualized on nitrocellulose sheets, as directed, by staining (10 min) with Ponceau S Red (10% v/v Ponceau S Red concentrate) and then destaining until the contrast was optimal (1-20 min) with water, or with 5% v/v acetic acid. The stain faded less rapidly after the nitrocellulose was removed from water. Stained nitrocellulose could be stored for several days (preferably in the dark, preferably at 4°C), but generally transfers were photographed and then immunostained. The PBS/Tween buffer (10mM PBS, 0.1% v/v Tween 20, pH 7.4) rapidly removed the Ponceau S Red stain from nitrocellulose sheets.

3.2.7.3 Immunoblotting after PAGE

Immunostaining proceeded, with only minor modifications, as described in Peers *et al*, 1993. Unless otherwise stated, all incubations were performed at room temperature, under gentle agitation. The PBS/Tween buffer was 10mM PBS containing 0.1% v/v Tween 20, pH 7.4.

Some nitrocellulose transfers were blocked, before incubation with the primary

probe. According to Vector stain literature, blocking is generally not required when biotinylated antibodies are used as primary probes. Some transfers were blocked using the Avidin/biotin blocking kit, as directed. Briefly, transfers were incubated for 15 min with Avidin in PBS/Tween; washed twice for 5 min with PBS/Tween; incubated for 15 min with biotin in PBS/Tween; and finally washed thrice for 10min in PBS/Tween. None of the alternative blocking conditions described in the Vector stain literature are discussed in this thesis. When unlabelled antibodies was used as primary probes, transfers were usually protein blocked, by incubation (60 min) in PBS/Tween containing Marvel skimmed milk (5% w/v) and then washed twice with PBS/Tween (5 min).

Transfers were incubated for 18 h with primary antibody in PBS/ Tween, then they were washed twice with PBS/Tween (15 min). The transfers were incubated (4h) with the secondary probe, at the recommended concentration, in PBS/Tween and finally washed thrice with PBS/Tween (15 min), then once with PBS (15 min) before visualisation. The visualisation method depended on secondary probe, three secondary probes were used:-

a) **Peroxidase conjugated anti-rat IgG antibodies** are generally recommended to be used at 0.02% v/v. Peroxidase stained proteins were visualized by the precipitation of the insoluble product of peroxidase catalyzed DAB oxidation. Transfers were incubated in PBS containing 0.6% v/v H_2O_2, 0.1% w/v DAB. The optimal contrast between specific and non-specific staining was usually achieved after 10-150 sec incubation. The reaction was stopped by washing the transfers thoroughly in water.

The sensitivity of DAB staining could be enhanced ten-fold using the nickel enhancement procedure (De Blas and Cherwinski, 1983). Transfers were incubated for 10 min in a prestaining solution which consisted of 10mM PBS containing 0.1% w/v DAB, 0.03% w/v ammonium nickel (II) sulphate [$(NH_4)_2Ni(SO_4)_2.6H_2O$] and 0.03% w/v cobalt (II) chloride [$CoCl_2$]), and then (10 min). Then the transfers were incubated for 10 min in a staining solution, consisting of prestaining solution plus 0.6% v/v H_2O_2. Both prestaining and staining solutions were freshly prepared then filtered though 0.45 μm nitrocellulose to remove undissolved DAB. Colour development was stopped by washing the transfers in water.

b) **Peroxidase conjugated Avidin** was used near the recommended concentration (0.1% v/v). Peroxidase stained proteins were visualized using nickel-enhanced oxidative DAB precipitation, as described above. Further enhancement was achieved by successive exposures (up to 3 sandwiches) to biotinylated goat (anti-Avidin) antibodies and peroxidase conjugated Avidin (Vector stain literature).

c) **^{125}I labelled Avidin** was used near 0.1% v/v. Immunostained protein bands were visualized by autoradiography (2 weeks, using Tritium sensitive film).

3.2.8 Enzyme-linked immunosorbent assay (ELISA) analysis

Samples were resuspended to 10 μg protein /ml in carbonate buffer (50mM sodium carbonate, 0.01% v/v Thiomersal, pH 9.6) and dispensed (250μl) into titretech plates. After 18 h incubation (4°C) plate wells were thrice washed gently with PBS/Tween (PBS, 0.1% v/v Tween 20, pH 7.4). The wells were blocked for 1 h in PBS/Tween at room temperature, and thrice washed with PBS/Tween. Relevant wells were filled (250μl) with PBS/Tween with or without primary

antibody cocktail (0.4% v/v) containing: 270 (titre 1.6nM, detects 200fmol nAChR); 290 (titre 27nM, detects 3380fmol nAChR); 297 (titre 0.3nM, detects 38fmol nAChR); 299 (titre 0.3nM, detects 38fmol nAChR). After a 4h incubation plate wells were thrice washed with PBS/Tween and filled (250μl) with PBS/Tween containing the secondary antibody (0.02% v/v). After a 4h incubation plate wells were washed thrice with PBS/Tween, then twice with PBS. The peroxidase was quantified colorimetrically (450 nm) by measuring the oxidation of Tetramethyl benzidine. Sodium acetate/citrate buffer (250μl of 50mM: 0.68% w/v sodium acetate, 0.0315% w/v citric acid) containing Tetramethyl benzidine [400μM, TMB], DMSO (1% v/v), H_2O_2 (0.005% v/v), pH 6.0, was added to each well, oxidation was stopped after 30min by the addition of 1.0M Sulphuric acid [50μl H_2SO_4].

3.2.9 Immunostaining material in free suspension

Specimens (P2b preparation) were immunostained in solution using a procedure developed from ELISA and prefixation immunostaining, two probes were used:-

a) mAbs recognized by peroxidase conjugated anti-rat IgG

b) biotinylated mAbs recognized by radiolabelled Avidin (cf section 3.2.7.3)

Samples were washed by dilution to 2% v/v with PBS, centrifugation (1,000g, 7 min, Sorval) and gentle resuspension using a pasteur pipette. When samples were blocked, the procedure described for nitrocellulose in section 3.2.7.3 was used. The sample was resuspended in PBS containing primary antibody probe cocktail (0.4% v/v) containing: 2 μl of 270 (titre 1.6nM, detects 1.6pmol nAChR); 2 μl of 290 (titre 27nM, detects 27pmol nAChR); 2 μl of 297 (titre 0.3nM, detects

0.3pmol nAChR); 2 μl of 299 (titre 0.3nM, detects 0.3pmol nAChR). After 18h incubation (4°C) under gentle agitation samples were twice washed (15 min) with PBS. The samples were resuspended with PBS containing the secondary probe (0.02% v/v for peroxidase conjugate, 1% v/v for radiolabelled Avidin) before a 4h incubation (room temperature). Samples were washed with PBS a recorded number of times and the concentration of secondary probe measured. The peroxidase content of a pellet was quantified colorimetrically (450 nm) in a Titretek multiscan reader by measuring the oxidation of 5AS. The pellet was resuspended (10% v/v) in PBS containing 0.2% w/v 5AS and 0.6% v/v H_2O_2, pH 7.4. After 30 min (room temperature) the reaction was stopped by the addition of 1/10 volume 1M NaOH. The radiolabelled Avidin content of a pellet was quantified using a gamma counter.

3.2.10 Immunoprecipitation of detergent solubilized nAChR using the Rogers sera

An immunoprecipitation protocol was used to measure the concentration of detergent solubilized nAChR. This method, summarized below, was described by Flores *et al* (1992), with further practical details provided by S.Rogers (person. comm.). Tissue preparation was prepared or resuspended (sections 3.2.1,3.2.2and 3.2.4) to approximately 20mg protein/ml in a Tris/salts/Triton buffer containing Tris (50mM), sodium chloride (120mM), potassium chloride (5mM), magnesium chloride (1mM), calcium chloride (2.5mM), Triton X-100 (0.5%v/v), pH 7.0. Unless otherwise stated, all incubations proceeded under gentle agitation provided by an end-over-end shaker. After a 2h incubation (4°C), and then centrifugation (48,000g, 30 min, Beckman), the detergent solubilized material (supernatant) was

diluted (to 0.25% v/v Triton) with a Tris/salts buffer which was identical to Tris/salts/Triton, but without Triton, pH 7.0. The solubilized nAChR preparation was dispensed into Eppendorf microfuge tubes (6 x 1ml) to which primary probe (0.125% v/v) was added. The radiolabelled ligand ± cold ligand were added (as section 3.2.6): ie [^{3}H]-cytisine plus water was added to determine total binding; [^{3}H]-cytisine and (-)-nicotine were added to determine non-specific binding. The material was incubated either at room temperature (2 h) or at 4°C (18 h) and then stripped (Shantz and Palmer, 1983) Pansorbin cells (10% v/v) were added. Pansorbin cells were stripped immediately prior to use, they were dispensed, in a uniform suspension, into Eppendorf microfuge tubes, centrifuged (10min, 10,000g, microfuge), the pelleted cells were resuspended (10% w/v) in stripping buffer containing Tris (50mM), NaCl (150mM), ß-mercaptoethanol (10% v/v), SDS (3% w/v), pH 7.2, and incubated at 95°C (30 min). Centrifugation, resuspension and incubation were repeated once. After centrifugation of the stripped cell suspension (10 min, 10,000g) the pellet was resuspended in one volume of the Tris/salts buffer. The mixture of stripped Pansorbin cells and antibody exposed material was incubated for 1 h (room temperature). The Pansorbin cell bound nAChR was immunoprecipitated by centrifugation in a vari-microfuge (10 sec, 1000g). The supernatant from each tube was rapidly removed by aspiration and the surface of each pellet gently washed once, by the rapid addition and then removal by aspiration of 1ml 10mM Tris containing 1mM EDTA, pH 7.5. The total time for centrifugation and washing of any sample was less than one minute. Each pellet was solubilized in 0.1M NaOH (100μl) containing sodium deoxycholate (3% w/v) and mixed, in a scintillation vial, with 5ml scintillant. Tritium levels were measured with the 1600 TR.

3.3 Results

3.3.1 Optimization of the [^{3}H]-nicotine binding assay

The method for measurement of [^{3}H]-nicotine binding, described by MacAllan *et al* (1988), was improved by using the Tris/HEPES buffer instead of 50mM Phosphate buffer during ligand incubation (Romm *et al*, 1990). As anticipated this buffer change reduced non-specific binding (figure 3.5), but caused no change in specific binding when attempts were made to detect [^{3}H]-nicotine binding to Triton X-100 detergent extracts of P2 membranes.

The results of performing the filtration assay upon buffer blanks is displayed in figure 3.6:-

a) at high Triton concentrations (>0.5% v/v Triton) there is a tendency for the values of both total and non-specific binding to rise, this result was frequently observed attempts were made to measure ligand binding at high Triton concentrations (Smillie, unpublished observations), the rise in measured binding correlates with a noticeable retardation in the passage of the blanks through the filter.

b) at Triton concentrations of 0.25% Triton or below the level of non-specific is similar to the level of non-specific binding to tissue preparations (figure 3.6)

c) there is considerable variation between replicate experiments, even though each measurement is made in triplicate, this means if n is low or if specific binding is low, the potential errors in specific binding measurement can be large: eg 100 cpm (see figure 3.6) is equivalent to around 2.5 fmol [^{3}H]-nicotine!

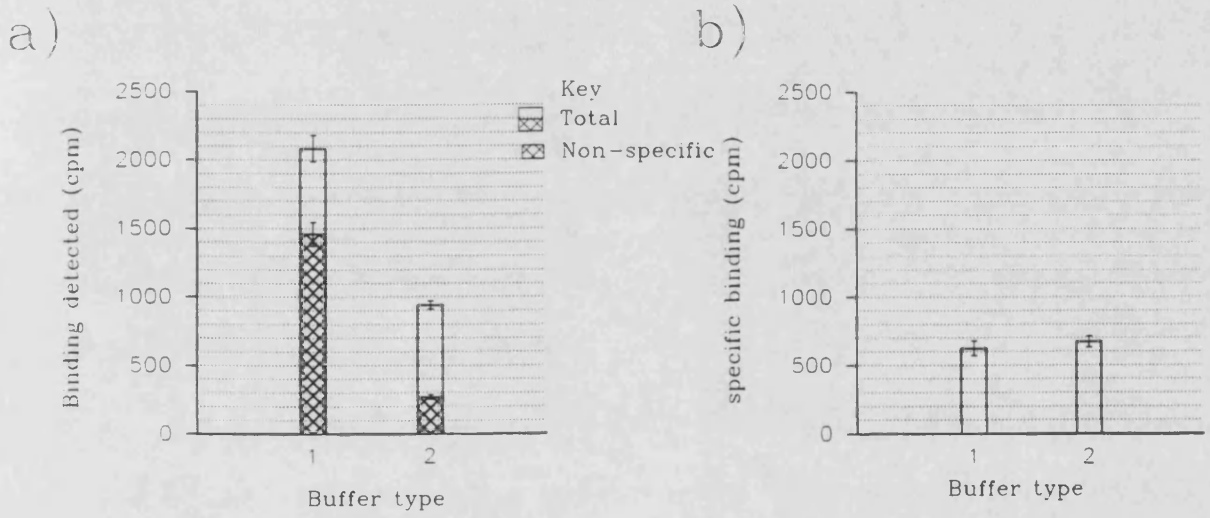
Figure 3.5 The effect of buffer composition upon [^{3}H]-nicotine binding

Buffer composition has an effect upon [^{3}H]-nicotine binding, figure 3.5 compares the effect of 2 buffers upon:-

a) Total and non-specific binding to a detergent extract of P2 membranes.

Protein concentration = 1mg/ml, Triton concentration = 0.05% v/v.

b) Specific binding to detergent extracts of P2 membranes as derived from the (a) according to the formula:- Total binding = Specific binding + Non-specific binding



The two buffers were:-

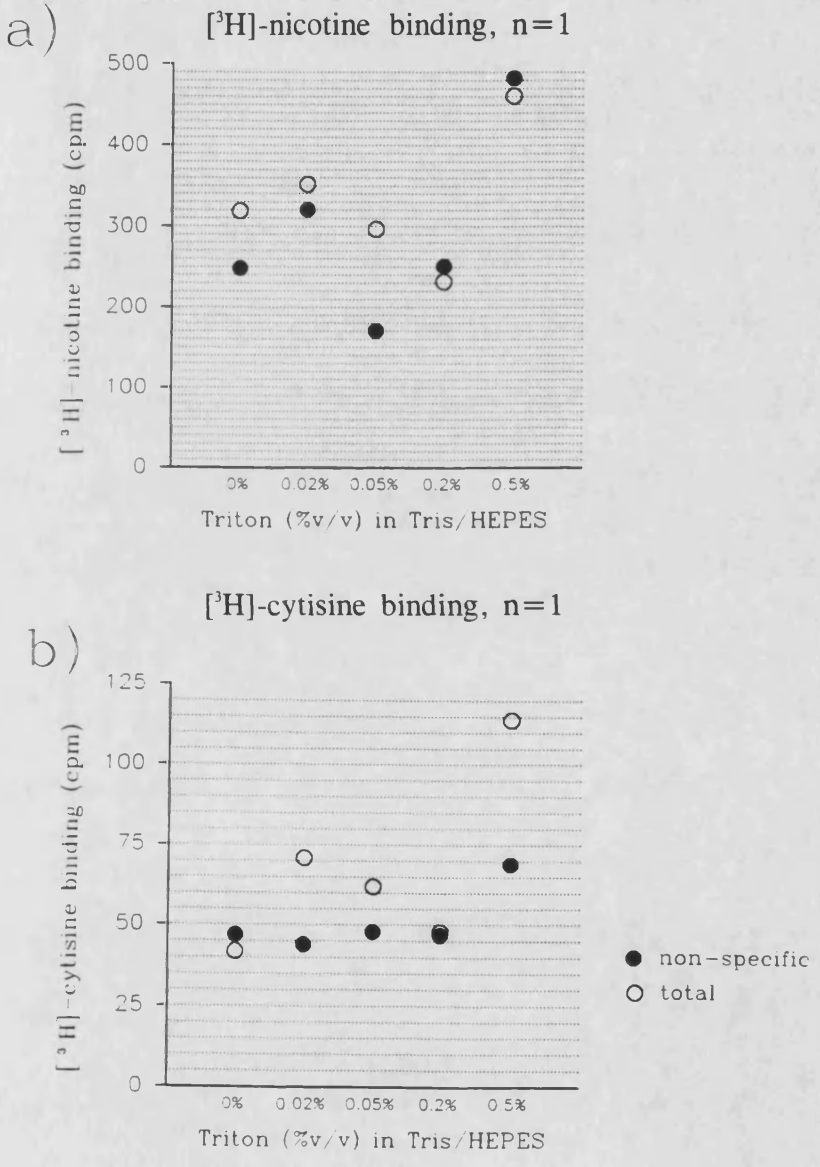
1) Phosphate buffer (50mM, pH 7.4), n = 3, standard error of the mean, SEM shown

2) Tris/HEPES buffer, n = 4, SEM shown

Specific binding using the phosphate buffer and the Tris/HEPES buffer was not significantly different at the 0.05 level (t-test, t=0.71, P=0.510)

Figure 3.6 Apparent binding to buffer blanks

Figure 3.6 displays the levels of total and non-specific ligand binding to buffer blanks, consisting of Tris/HEPES buffer containing Triton X-100 at various concentrations, binding was measured in triplicate (n=1) by the filtration assay. These graphs demonstrate the variation in binding measurements and also the effect of Triton X-100.



There seemed to be a tendency to detect a small level of apparent specific binding:-

a) mean specific [³H]-nicotine binding for all 5 blanks of graph (a) = 37.4 (SEM = 22)

b) mean specific [³H]-cytisine binding for all 5 blanks of graph (b) = 16.4 (SEM = 8.9)

The binding of [^{3}H]-cytisine (Pabreza *et al*, 1991) using the same conditions, except for incubation times and temperature (section 3.2.6)is also shown in figure 3.6. The comments (a), (b) and (c) above can also be applied to [^{3}H]-cytisine binding except the level of radioactivity is lower. (36 cpm was equivalent to around 1.4 fmol [^{3}H]-cytisine).

Some effects upon [^{3}H]-nicotine binding of the storage (4°C) of the extracts and of the Tris/HEPES buffer are displayed in figure 3.7:-

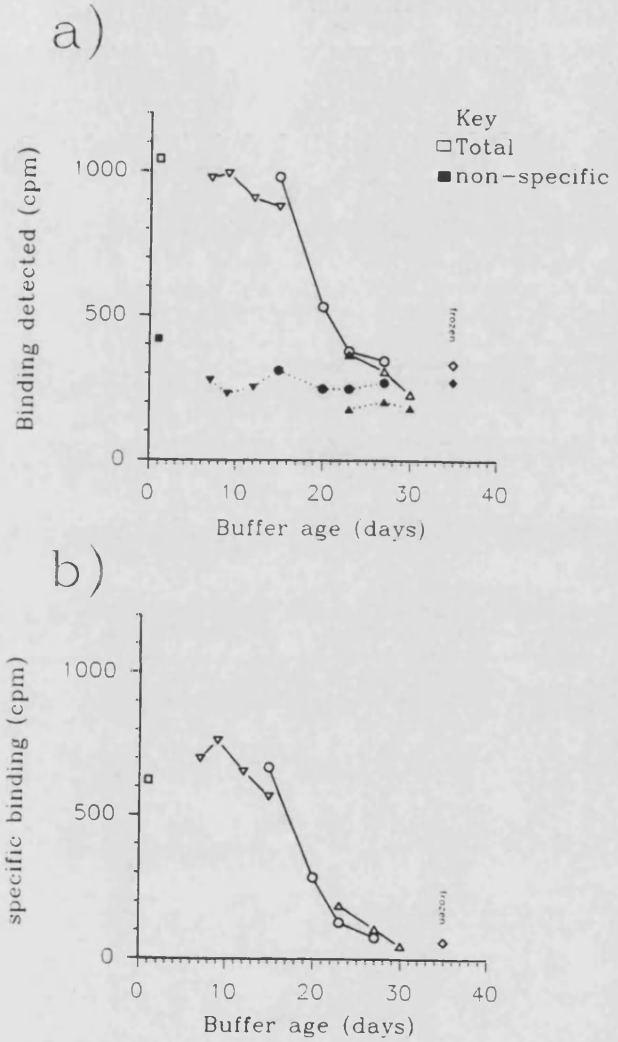
a) As the Tris/HEPES buffer ages beyond about 10 days the level of detectable specific binding falls. The buffer was stored at 4°C and used within 7 days of preparation. Parallel studies (M.W. Stephens, within the laboratory) indicated that buffer could be stored indefinitely at -30°C, and, after thawing, it could be treated as if freshly prepared.

b) When detergent extracts of P2 membranes were stored by freezing at -30°C all specific activity was lost, however the loss of specific activity was far more gradual when stored at 4°C. It was apparent that there was negligible loss of activity during the first 48-72h of storage at 4°C, when fresh Tris/HEPES buffer was used; detergent extracts were always freshly prepared and generally were not incubated for more than 24h.

The binding of [^{3}H]-nicotine to detergent extracts of P2 membranes by filtration under constant Triton concentration was found to be proportional to protein concentration, up to 3.5mg/ml protein (figure 3.8). The problems of handling very concentrated protein solutions prior to the dilution step placed an upper practical limit for extract concentration of around 2mg/ml protein during filtration in

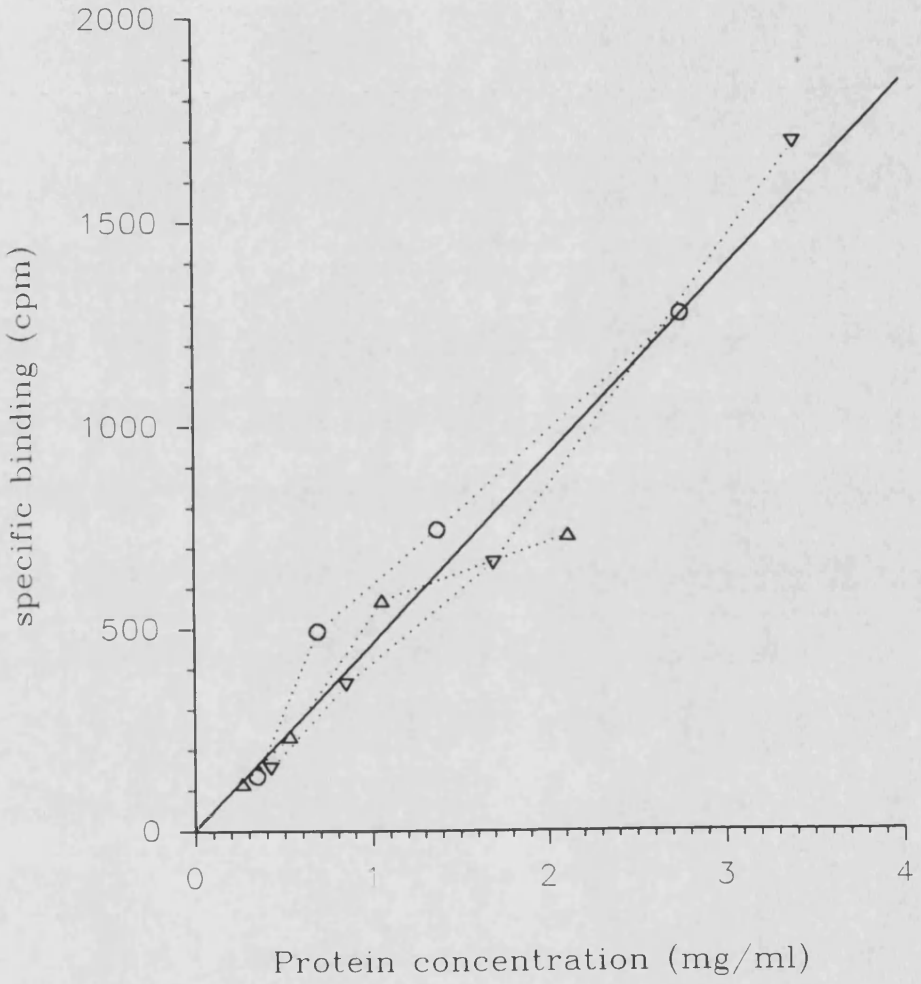
Figure 3.7 The effect of storage upon [^{3}H]-nicotine binding to a detergent extract of P2 membranes

Figures 3.7.a(total and non-specific binding) and 3.7.b(specific binding) summarize the effect on [^{3}H]-nicotine binding filtration assays of increasing buffer age and increasing preparation age.



Tris/HEPES buffer was prepared and stored at 4°C, in the dark. As the buffer aged, 3 detergent extracts of P2 membranes were prepared, these were also stored at 4°C, in the dark. The level of [^{3}H]-nicotine binding to extracts at 0.05% v/v Triton, and protein concentration = 1mg/ml was measured using the original buffer at various times after preparation. The extract marked "Frozen" was prepared with fresh buffer; stored at -30°C, in the dark, for 7d; and then assayed using fresh buffer. The ligand binding to all samples was measured in triplicate. Each preparation was represented with a different symbol. For each point displayed, n=1.

Figure 3.8 The effect of protein concentration upon [^{3}H]-nicotine binding to detergent extracts of P2 membranes at constant Triton concentration



The pooled results of 3 dilution experiments at constant Triton concentration (0.1% v/v) are shown in figure 3.8. The specific binding was calculated from triplicate measurement of total and of non-specific binding. Each dilution series was represented with a different symbol, for each point n = 1.

immunoisolation experiments. This is a convenient protein concentration, because nicotine binding in the absence of Triton is proportional to protein concentration up to 2mg/ml protein (Lippiello and Fernandes, 1988; MacAllan *et al*, 1988; Wonnacott, person. comm.; Smillie unpublished observations).

3.3.2 Ligand binding to synaptic junctional complexes

Synaptic plasma membranes (SPM) were prepared from brain tissue (6 rat brains, 8.5g) and then solubilized and fractionated to yield synaptic junctional complexes (SJC: 0.7mg) and solubilized synaptic plasma membranes (SPM-SJC: 0.6mg) were prepared in about 36 h (section 3.2.3) and assayed for nicotine binding (section 3.2.6). These preliminary experiments indicated that high affinity [^{3}H]-nicotine binding sites were not enriched in SJCs or the solubilised SPM-SJC preparations (figure 3.9). Calculations show that 1 rat brain (1.2g) produces 8mg P2b (720fmol nAChR) which produces 0.12mg SJC (20.5fmol nAChR) and 0.1mg SPM-SJC (20fmol nAChR). Each of these 2 receptor populations represent about 2.8% of the high affinity [^{3}H]-nicotine binding nAChRs measured in P2b preparations. These results should be interpreted with some caution, because the protein concentration and ligand binding (cpm) were low, but they show that high affinity [^{3}H]-nicotine binding sites are present at the synaptic junction (SJC) and away from the synaptic junction (SJC-SPM) in synaptosomes. It was not considered a worthwhile use of resources to pursue these experiments further, a precise determination of the nAChR content of SJC or SPM would be difficult to achieve and the additional information gained would be negligible.

Figure 3.9 The binding of [^{3}H]-nicotine to synaptic junctional complexes (SJC) and synaptic plasma membranes (SPM)

a)

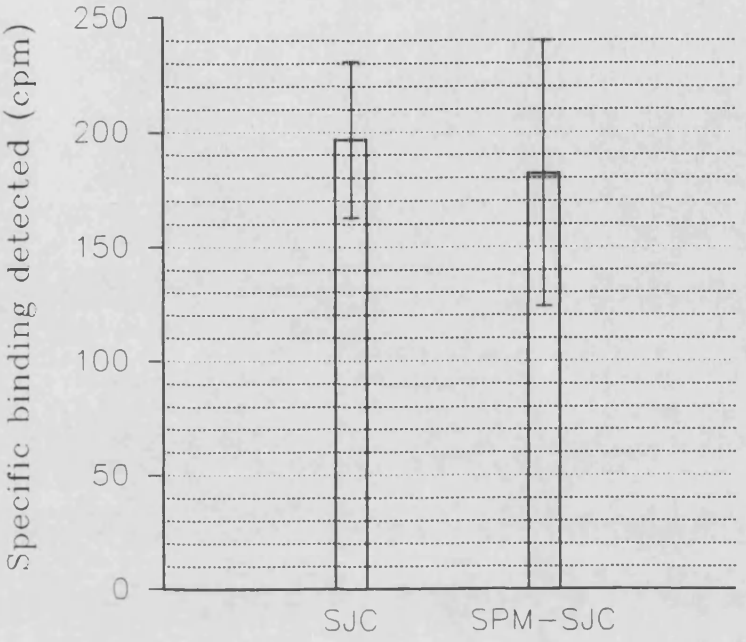


Figure 3.9.a displays the results of experiments to determine specific binding of [^{3}H]-nicotine to SJC (n=4) and SPM (n=2) in cpm. SEMs shown.

b)

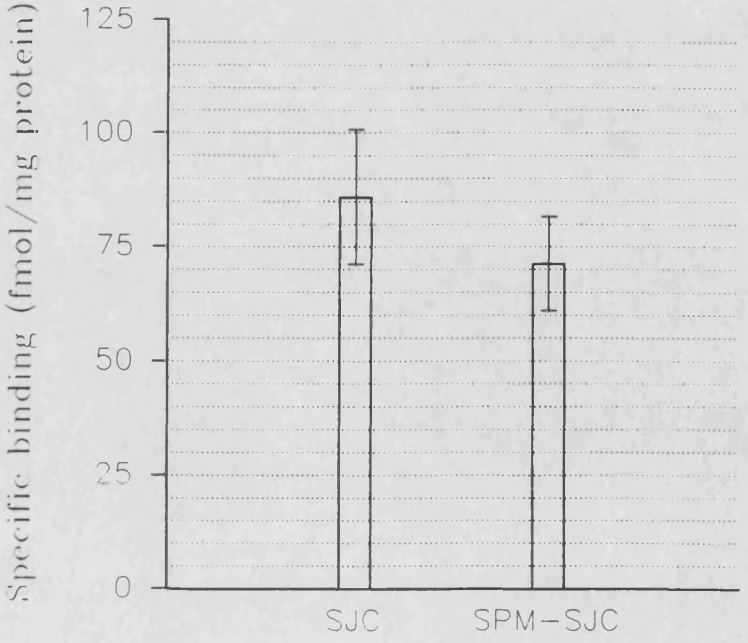


Figure 3.9.b displays the results of experiments to determine specific binding of [^{3}H]-nicotine to SJC (n=4) and SPM (n=2) in fmol/mg protein. SEMs shown.

3.3.3 Subcellular localisation using discontinuous density Percoll gradients: optimization of conditions

An examination of the distribution of nAChR subtypes which bind [^{3}H]-nicotine at high affinity was compromised in previous experiments by the presence of Percoll: Percoll binds [^{3}H]-nicotine in a manner which mimics specific binding to the receptor (Wonnacott and Wilkie, 1991). This observation was confirmed by measuring [^{3}H]-nicotine and [^{3}H]-cytisine binding by filtration (section 3.2.6) to buffer blanks containing Percoll (figure 3.10). The specific binding of [^{3}H]-nicotine (2009 fmol/ml Percoll) is far greater than the specific binding of [^{3}H]-cytisine (216 fmol/ml Percoll) to Percoll: both represent unacceptably high levels. The value of B_{max} for specific [^{3}H]-nicotine binding is similar to the value of B_{max} for specific [^{3}H]-cytisine binding (Lippiello and Fernandes, 1986, 1888; Pabreza *et al*, 1991; further references in section 1.3). The concentration of the $\alpha 4\beta 2$ isoform in Percoll fractions was measured using [^{3}H]-cytisine binding, because its binding is less compromised by the presence of Percoll. The degree of ligand binding to Percoll seemed largely unaffected by freezing or short term storage (4°C) of the filtered Percoll solution.

Three approaches were taken try to minimize the effect of Percoll upon [^{3}H]-cytisine binding:-

a) Repeated washing by centrifugation. P2 membranes were extensively centrifugally washed before the measurement by [^{3}H]-cytisine binding. Percoll forms a very dense gelatinous precipitate during high speed centrifugation, which is harder to resuspend than tissue pellets. It was possible to remove all/most of the Percoll, this was confirmed by binding assay (figure 3.11, preparation 1),

Figure 3.10 The binding of [^{3}H]-cytisine and [^{3}H]-nicotine to Percoll suspensions

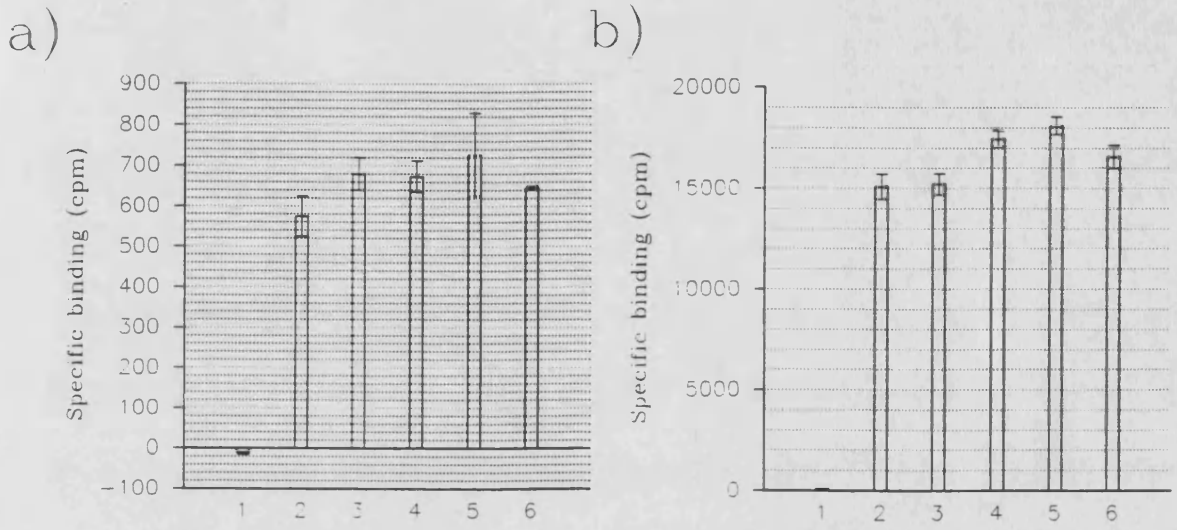


Figure 3.10.a shows [^{3}H]-cytisine binding and figure 3.10.b shows [^{3}H]-nicotine binding to:-

1) 50:50 Tris/HEPES:sucrose, n=3

2) 50:50 Tris/HEPES:23% Percoll in sucrose, n=4. Fresh Percoll.

3) 50:50 Tris/HEPES:23% Percoll in sucrose, n=2. Percoll suspension frozen after preparation.

4) 50:50 Tris/HEPES:23% Percoll in sucrose, n=2. Percoll suspension stored for 3d, at 4°C, after preparation.

5) 50:50 Tris/HEPES:23% Percoll in sucrose, n=2. Percoll suspension stored for 4d, at 4°C.

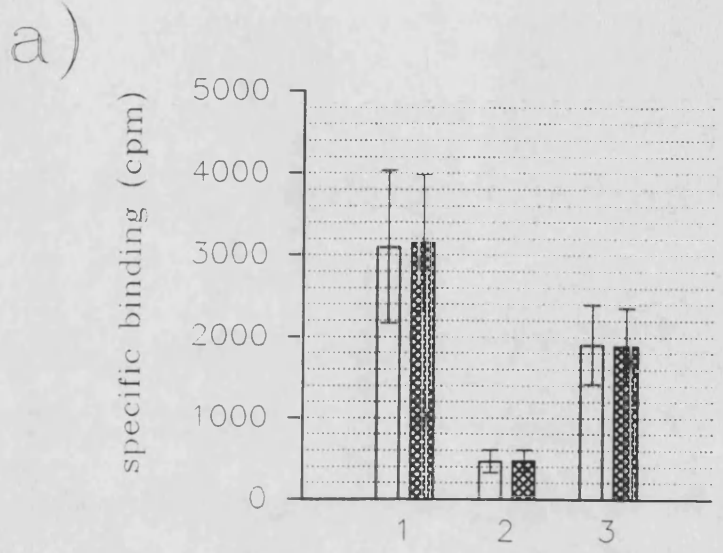
6) 50:50 Tris/HEPES:23% Percoll in sucrose, n=2. Percoll suspension stored for 5d, at 4°C.

SEMs shown. Mean specific binding to Percoll suspension:-

a) 216 fmol [^{3}H]-cytisine/ml of original (ie 100%) Percoll suspension

b) 2009 fmol [^{3}H]-nicotine/ml of original Percoll suspension

Figure 3.11 **The binding of [^{3}H]-cytisine to P2 preparations in the presence and absence of Percoll**



Controls were performed to determine if methods could be established, which minimized the effect of Percoll upon [^{3}H]-cytisine binding. In figure 3.11.a two types of preparation are displayed:- P2 preparation containing Percoll (▨), P2 preparation containing no Percoll (▭). This figure compares 3 assays:-

1) Filtration of heavily washed P2 membranes. n=3. 0.5ml samples.

2) Filtration of the detergent extracts. n=4. 0.5ml samples.

3) Immunoprecipitation of the nAChR from detergent extracts using Rogers antisera and Pansorbin cells. n=4. 1.0ml samples.

Note, in these experiments care was taken to match the protein concentration of Percoll/no Percoll pairs, however figure 3.11.a displays raw data, which has not been corrected for protein concentration, so there is variation between experiments. SEMs shown.

b)

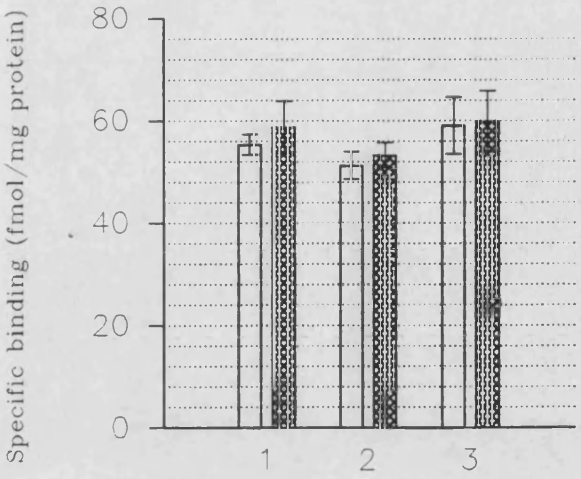


Figure 3.11.b displays the same data as figure 3.11.a, except specific binding is expressed in terms of fmol/mg of protein, corrected for detergent concentration in the case of measurement approach (2). Statistical analysis (t-test of all pairs) indicates (not shown) that there is no difference at the 0.05 level between each Percoll/no Percoll pair. SEMs shown.

c)

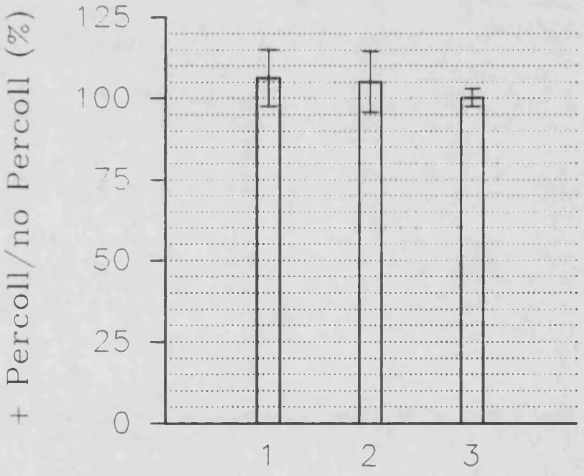


Figure 3.11.c displays the relationship between [^{3}H]-cytisine binding to pairs of P2 membranes containing and not containing Percoll, ie for each experiment in each approach to binding the value of:

$$\frac{\text{P2 preparation containing Percoll} \times 100\%}{\text{P2 preparation not containing Percoll}}$$

is determined. Statistical analysis (t-test of all pairs) indicates (not shown) that there is no difference at the 0.05 level between (1), (2) and (3). SEMs shown.

however the resuspension of tissue pellets was very difficult without the parallel resuspension of Percoll pellets. This approach was unsatisfactory, because any potential Percoll resuspension would become a significant factor when examining samples containing low protein concentrations.

b) Filtration of detergent extracts. The problems of pellet resuspension were avoided by performing a detergent extraction upon P2 membranes (± Percoll). No washing was required because all the Percoll and non-solubilized material formed a pellet during centrifugation, the filtration assay was performed upon the solubilized material (figure 3.11, preparation 2). The effects of Triton upon the filtration assay were reduced by dilution of extracts (0.5% v/v to 0.25% v/v Triton), however the variance associated with low specific binding using the filtration assay to preparations could not be avoided, protein concentration was lowered (yield 28%) by extraction and Triton concentrations were relatively high.

c) Immunoprecipitation of detergent extracts. Immunoprecipitation (using antibodies against α4 and ß2 donated by S. Rogers) of detergent solubilized nAChRs which had been exposed to [^{3}H]-cytisine (see section 3.2.10) was found to be unaffected by the presence of Percoll in the original preparation (figure 3.11, preparation 3). Specific binding of [^{3}H]-cytisine, measured by the precipitation assay (section 3.2.10), approaches the level of binding to preparations in the absence of Triton by the filtration assay indicating that this approach to measuring [^{3}H]-cytisine binding was unaffected by Triton. Non-specific binding was far higher (10 times: 1000cpm cf 100cpm) than non-specific binding measured in filtration assays and so this approach was less accurate than (b), especially at low protein concentrations, because of the relatively high non-specific to specific binding ratio (Lunt, 1987; Yamamura *et al*, 1985)!

In summary, the best approach to measuring nAChR in Percoll fractions was the filtration (b) of detergent extracts. The immunoprecipitation assay (c) is not as accurate as the filtration of detergent extract assay (b), however the results of this assay do reinforce the proposition that the nAChR isoform which is measured is the $\alpha 4\beta 2$ subtype.

3.3.4 Subcellular localisation using discontinuous density Percoll gradients: Examination of Percoll gradients

The S1 fraction prepared from rat brain cortex was separated using discontinuous density Percoll gradients (section 3.2.4). Figure 3.12 displays the protein distribution in fractions from Percoll gradients. Figure 3.13 displays the results of [^{3}H]-cytisine binding to fractions from Percoll gradients, measured by the filtration assay. No washing was performed after resuspension to 11 ml. Figure 3.14 displays the results of [^{3}H]-cytisine binding to detergent extracts of fractions from Percoll gradients (section 3.2.4),measured by the immunoprecipitation assay. The protein recovery during detergent extraction was approximately 28% in all fractions. The specific binding to F5 cannot be negative as indicated. The recovery of [^{3}H]-cytisine binding proteins was unexpectedly high considering the overall level of protein recovery. Figure 3.15 displays the results of [^{3}H]-cytisine binding to detergent extracts of fractions from Percoll gradients (section 3.2.4),measured by the filtration assay. The protein recovery during detergent extraction was approximately 28% in all fractions. The preparations were concentrated 4 fold before extraction. These experiments indicate that nAChRs are concentrated in fractions F4 and F5, but the bulk of nAChRs were found in fraction F2. These results are interpreted and related to previous studies in section 3.4.3.

Figure 3.12 **The distribution of protein down a discontinuous density Percoll gradient of S1 preparation**

a)

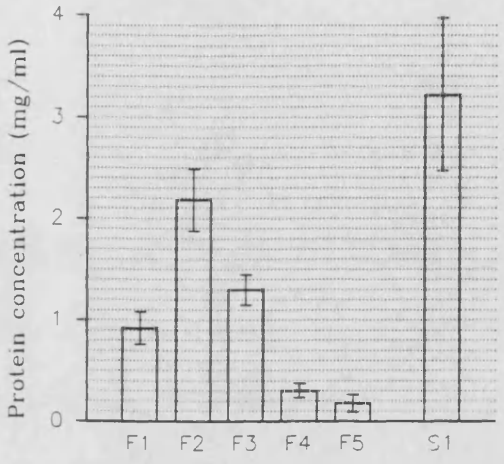


Figure 3.12.a shows the protein concentration of S1 (applied volume 20ml) and of the fractions (resuspension volume 11ml) from Percoll gradients (10 parallel gradients). n=4 throughout, SEMs shown.

b)

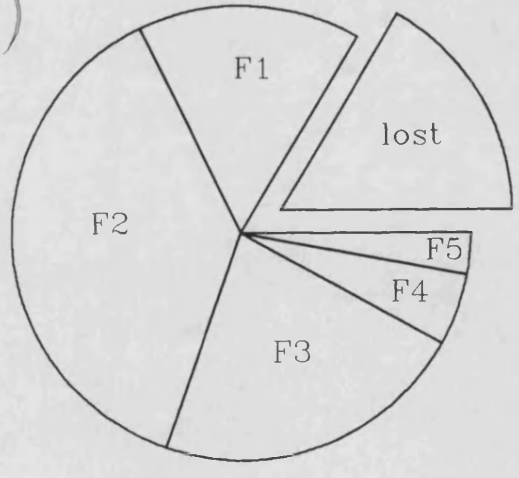


Figure 3.12.b displays total protein recovery (where S1 was 100%):- F1 (15.7%), F2 (37.3%), F3 (22.2%), F4 (5.1%), F5 (3.0%), so the overall protein recovery was 83.4%.

Figure 3.13 **The distribution of [^{3}H]-cytisine binding down a discontinuous density Percoll gradient measured using the filtration assay (no detergent extraction)**

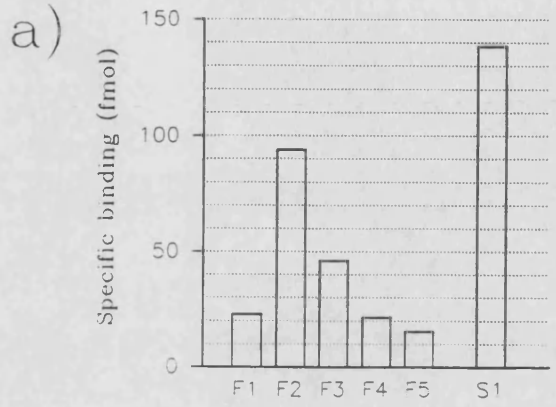


Figure 3.13.a displays the total specific binding of the S1 applied to Percoll gradients and of the resulting fractions. n = 1 throughout.

Figure 3.13.b should display the recovery of ligand binding proteins (where S1 was 100%):- F1 (16.5%), F2 (67.9%), F3 (33.4%), F4 (15.5%), F5 (11.1%), so the apparent total recovery of "ligand binding protein" was 144.4%! Clearly a pie chart is not meaningful.

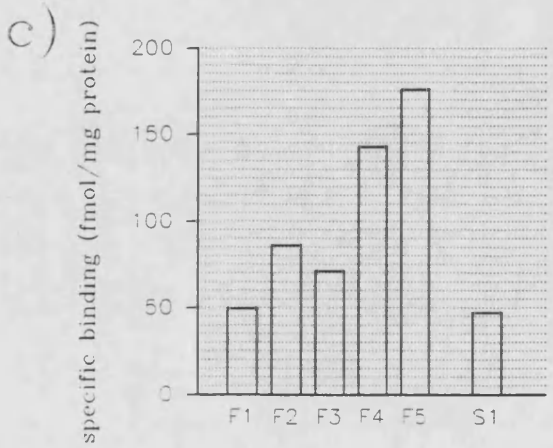


Figure 3.13.c displays apparent concentration of specific [^{3}H]-cytisine binding proteins per mg of total protein present. n = 1 throughout.

Figure 3.14 **The distribution of [^{3}H]-cytisine binding down the discontinuous density Percoll gradient measured using the immunoprecipitation assay**

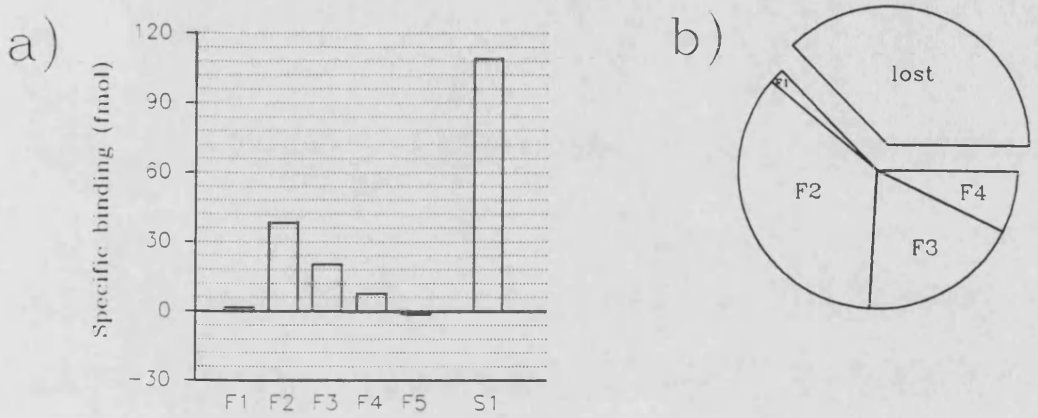


Figure 3.14.a displays the total specific binding of the S1 applied to Percoll gradients and of the resulting fractions as determined by the immunoprecipitation assay using Rogers anti-sera and Pansorbin cells. n = 1 throughout.

Figure 3.14.b displays the recovery of ligand binding proteins (where S1 was 100%):- F1 (1.7%), F2 (35.2%), F3 (18.6%), F4 (7.2%), F5 (-1.1%), so the apparent total recovery of ligand binding protein was 62.7%

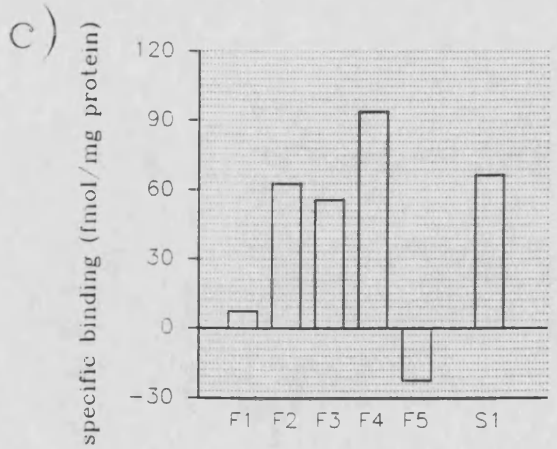


Figure 3.14.c displays apparent concentration of specific [^{3}H]-cytisine binding proteins per mg of total protein present. n = 1 throughout.

Figure 3.15 **The distribution of [^{3}H]-cytisine binding down the discontinuous density Percoll gradient, detergent extracts measured using the filtration assay**

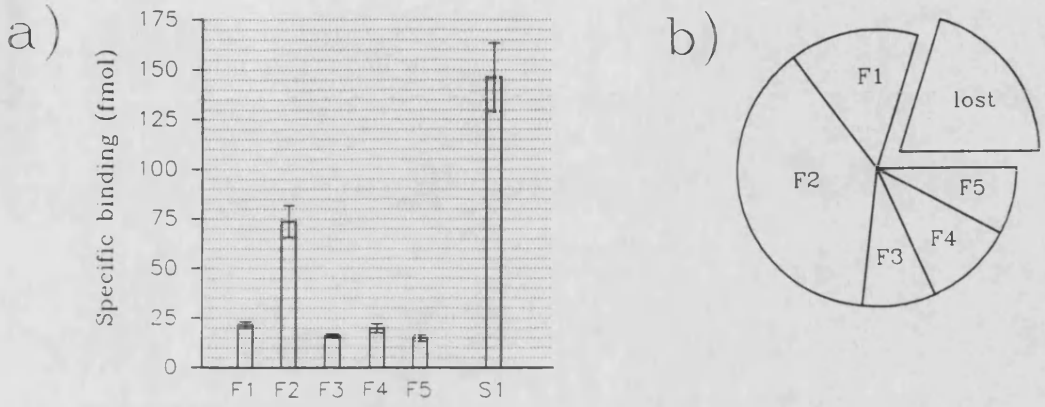


Figure 3.15.a displays the total specific binding to detergent extracts of the S1 applied to Percoll gradients and of the resulting fractions. n=5 throughout, SEMs shown.

Figure 3.15.b displays the recovery of ligand binding proteins (where S1 was 100%):- F1 (14.8%), F2 (38.2%), F3 (8.5%), F4 (10.5%), F5 (7.9%), so the apparent total recovery of ligand binding protein was 79.9%

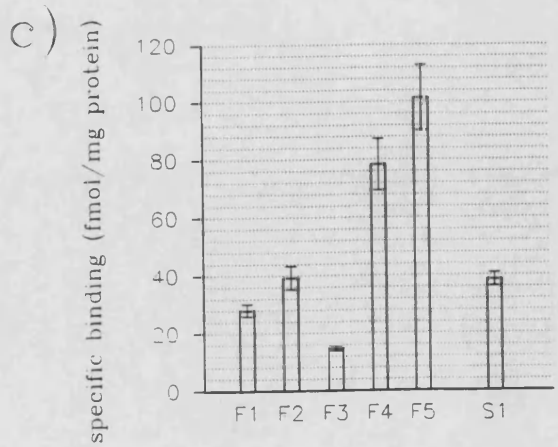


Figure 3.15.c displays apparent concentration of specific [^{3}H]-cytisine binding proteins per mg of total protein present. n=5 throughout, SEMs shown.

3.3.5 Polyacrylamide gel electrophoresis and immunoblotting

PAGE was used in two ways:-

a) To estimate the concentration of proteins,

b) To immunostain nAChRs.

3.3.5.1 Protein concentration

The concentration of one protein type (IgG), in samples which did not contain polypeptides with the same mobility on the PAGE gels (eg BSA), was estimated by running a serial dilution of the sample down a polyacrylamide gel (section 3.2.5.2). Plate 29 demonstrates the use of this method to estimate the protein concentration of a Lindstrom monoclonal antibody. The protein concentration of Lindstrom monoclonal antibodies is shown in table 2.1, the same technique was used to verify the concentration of biotinylated Lindstrom antibodies.

3.3.5.2 Immunostaining

The optimal protein concentration for PAGE differs with sample composition and gel size. An appropriate sample dilution had to be found, before proceeding with immunostaining. The optimal concentration of P2b samples (20μl) using the minigel system as described in section 3.4.1 was found to be 1.5 mg protein/ml (plate 30), this concentration and volume was used for immunostaining trials. The concentration of the $\alpha 4\beta 2$ in P2b samples was 45 fmol/mg protein (section 4.4.1.1, 5.3.4.1), thus each lane contains 1.4 fmol (or 0.34 ng) of nAChR.

Antibody biotinylation and preliminary immunostaining work, using biotinylated

Lindstrom antibodies, detected using the Vector stain system was performed, under my supervision, by C.Billington (1992, unpublished: fourth year project report). The subunits of immunopurified nAChR were detected by immunoblot analysis using biotinylated Lindstrom mAbs detected by the peroxidase-conjugated Avidin (Billington, 1992, unpublished).

The results of immunostaining experiments using Lindstrom antibodies for the direct recognition of nAChR in P2b preparations, without preliminary receptor purification, are summarized in plates 31, 32 and 33. These plates show Western transfers of lanes from PAGE of P2b in parallel with dot-blot controls (figure 3.16) using the three approaches to immunostaining with Lindstrom antibodies (section 3.2.7.3).Dot blot controls provided the following information:-

a) The biotinylated mAbs and the mAbs were recognized by the peroxidase conjugated anti-rat IgG antibodies.

b) The biotinylated mAbs were recognized by the peroxidase conjugated Avidin and by ^{125}I-Avidin, but neither recognized mAb cocktails which had not been biotinylated.

c) There was a low level non-specific tissue recognition by all secondary probes which increased with secondary probe concentration, however the majority of the dot coloration seen in the photographic plates was due to the slight colour of the P2 preparation itself.

d) Goat anti-(rat IgG) antibodies were not recognized under any of the conditions investigated.

Immunoblotting PAGE transfers was not sensitive enough to detect the α4 or ß2

Figure 3.16 The layout of dot blot controls

Dot blot controls were placed at the top of each nitrocellulose strip representing one well from PAGE experiments. The strips were then immunostained.

Figure 3.16a shows the layout for plates 31 and 32,

Figure 3.16b shows the layout for plate 33

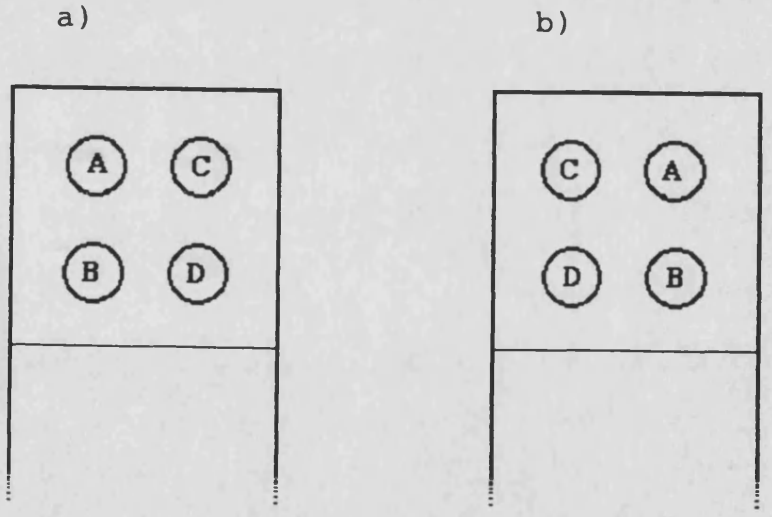
Controls:-

A biotinylated mAb cocktail (5μl)

B P2 membranes (5μl)

C Goat (anti-rat IgG) antibody (5μl)

D mAb cocktail (5μl of 1/20)



subunits in the P2b preparation. There was some non-specific staining, for example when secondary probes were used in excess, but the following observations indicated the absence of specific staining:-

a) the banding pattern after immunostaining was the same in the presence and absence of primary probe (plate 34)

b) the banding pattern after immunostaining was the same regardless of which Lindstrom antibody was used as primary probe (plate 35, 36 and 37)

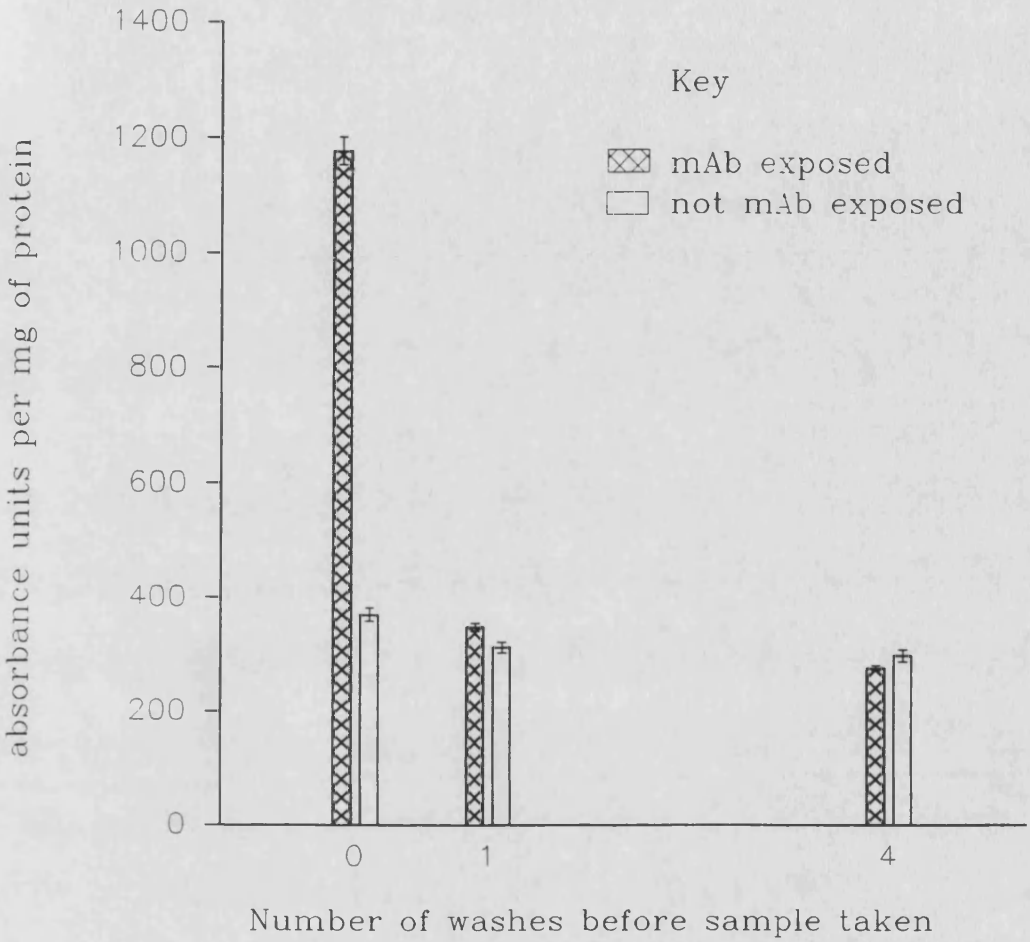
c) the molecular mass of the bands which were visible after immunostaining did not correspond to the molecular mass of the α4 (79kD) or the ß2 (51kD) subunits detected by immunoblotting immunopurified receptors (Whiting *et al*, 1987c; Whiting and Lindstrom, 1987).

These experiments were performed in triplicate and a variety of enhancement and blocking conditions were tried for each approach (only a representative example is shown), but each of the three approaches to immunostaining nAChRs directly in tissue preparations using Lindstrom mAbs were unsuccessful.

3.3.6 Indirect nAChR detection by ELISA

An attempt was made to use ELISA to detect nAChRs in synaptosome preparations. Figure 3.17 displays a representative experiment: mAb detection was demonstrated (no washing), but the non-specific signal (controls) was high and there was no detectable specific nAChR signal (rat IgG in washed samples). Calculation indicated that if specific recognition had occurred, it probably would not have been detectable using this approach to ELISA: using the recommended protein concentration for samples (10μg/ml), each microtitre well contained

Figure 3.17 **ELISA measurement of mAb binding to P2b**



Synaptosome preparations (P2b) were exposed to PBS ± mAb (0.4% v/v cocktail) then washed a recorded number of times. Samples were taken before each wash and the level of rat IgG measured by ELISA (5AS oxidation, 30 min). SEMs shown, n=4. Statistical analysis (t-test) of ELISA measurements indicated: after no washes ± mAb exposure samples differ at the 0.05 level (t=30), after 1 wash ± mAb exposure samples differ at the 0.05 level (t=3), but do not differ at 0.025, and after 4 washes ± mAb exposure samples do not differ at the 0.05 level.

approximately 0.07 fmol (17 pg) of nAChR. If one assumes each receptor was recognized by two antibody molecules, then the detection of this specific staining would cause an increase of 0.0025 Absorbance units per ELISA well, ie 1% of the non-specific staining. Experiments were performed to reduce the level of non-specific staining, they are not shown. The level of non-specific staining was reduced in these experiments, but the variation between samples was considerable. The use of ELISA was not pursued, because this approach was judged to be too insensitive to measure nAChRs from synaptosome preparations without prior immunopurification (section 3.4.5), but it initiated the use of potentially more sensitive immunostaining approaches described in section 3.3.7.

3.3.7 Immunostaining tissue in free suspension

Synaptosomes were immunostained in free suspension using a procedure based on ELISA and prefixation immunogold staining (section 3.2.9). This technique had 3 advantages over ELISA:-

a) There was a lower probability of non-specific staining, because of there was no matrix which might adsorb proteins.

b) More sample, containing more nAChR, can be immunostained increasing the probability of detection.

c) Large volumes of washing vehicle can be used increasing washing efficiency.

Whilst its advantage over prefixation immunogold staining related to the nature of the secondary probe:-

a) Probe dissociation was very unlikely (Hayat, 1989a, b; Williams, 1977b)

b) Detection was easy on a macroscopic scale.

Two types of secondary probe were used in attempts directly to immunostain nAChR in a tissue preparation in free suspension (section 3.2.9).

When peroxidase conjugated anti-rat IgG antibodies were used to immunostain tissue in free suspension, anomalous results were found. Figure 3.18a shows how the level of 5AS oxidation caused by controls (ie no mAb exposure) rises with samples taken from each successive wash after exposure to the secondary probe. The same trend was seen in samples which had been exposed to mAbs (Figure 3.18b). No specific staining was apparent in these experiments. Section 3.4.6 describes why this approach was unreliable, initiating the use of an alternative secondary probe (^{125}I-Avidin).

The use of ^{125}I-Avidin as a secondary probe for immunostaining avoided the false-signal problem encountered using the peroxidase conjugated anti-rat IgG antibodies as a secondary probe. In both control and cocktail exposed preparations some non-specific staining was measured (about 0.1 fmol of ^{125}I-Avidin per mg of protein). With each wash, between exposure to secondary probe and measurement, the level of non-specific staining fell equally in both preparations (figure 3.19). However, despite the potential sensitivity of this approach, no specific staining was measured in tissue preparations which had been exposed to a biotinylated monoclonal cocktail relative to control (no monoclonal exposure) preparations. Section 3.4.7 describes the interpretation of this result.

Figure 3.18 **Immunostaining tissue in free suspension using peroxidase conjugated probes to detect mAbs**

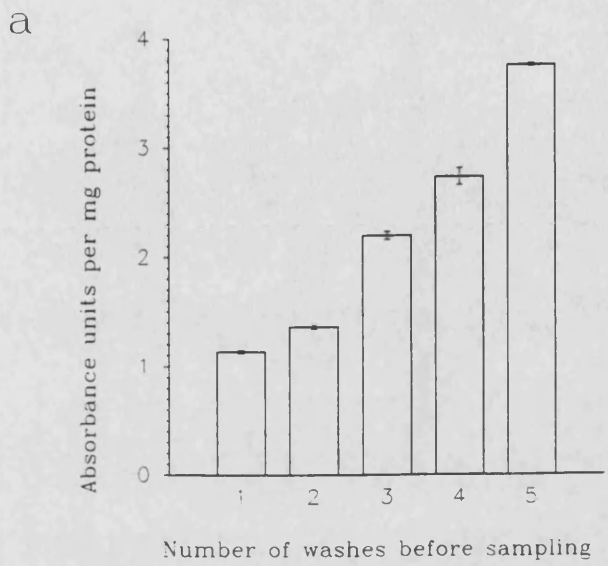


Figure 3.18.a displays immunostaining results for control P2b preparations, which had not been exposed to mAb cocktails prior to washing (1000g, 10min, diluted 2%v/v with PBS) and mAb detection (as section 3.2.9). n=3, SEMs shown.

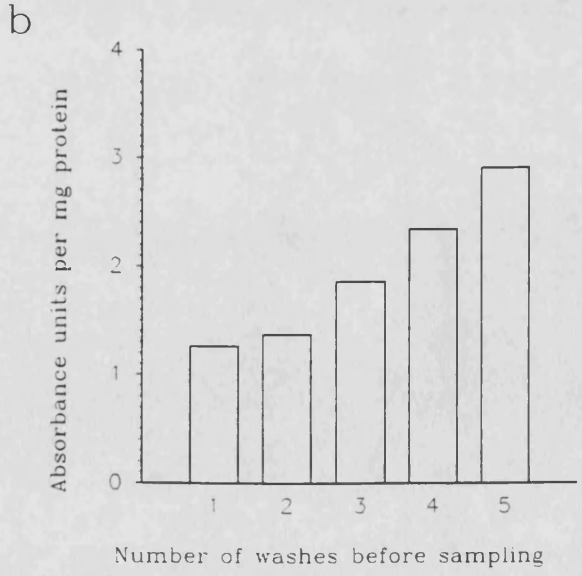
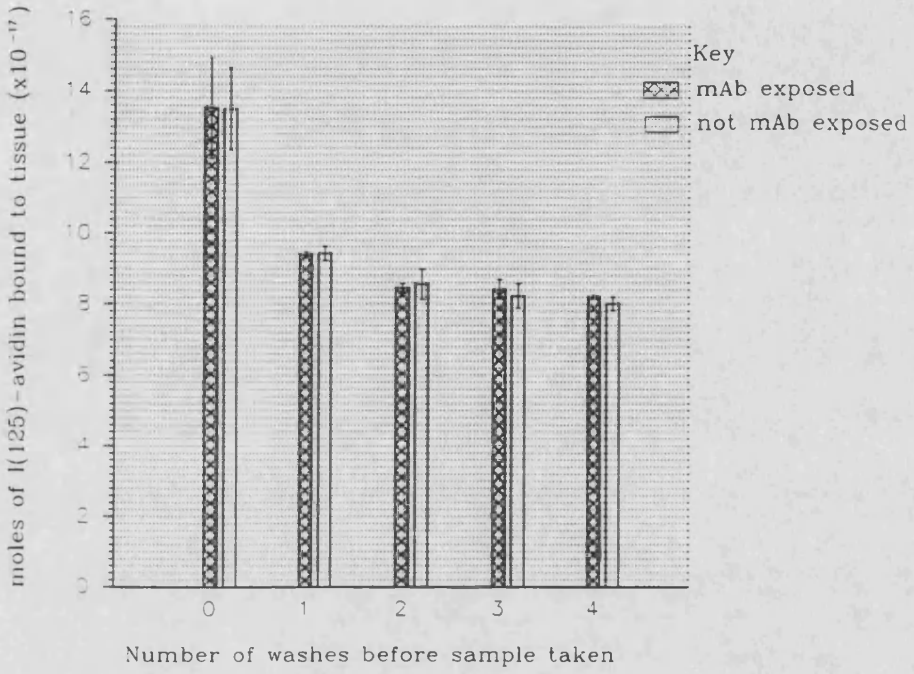


Figure 3.18.b displays immunostaining results for mAb exposed P2b preparations (washing 500g), n=1.

Figure 3.19 **Immunostaining tissue in free suspension using a ^{125}I-Avidin probe to detect mAb binding**



P2b preparations were exposed to PBS ± mAb (0.4% v/v cocktail), then to ^{125}I-Avidin (section 3.2.9). Note 1% v/v ^{125}I-Avidin was 250 x 10^{-17} mol ^{125}I-Avidin/mg protein in the P2b preparation. The preparation was washed by centrifugation (1,000g, 10min, preparation 2%v/v in PBS) a recorded number of times, 3 samples were taken before each wash. The pellets from centrifugation of the samples (1,000g, 5min, microfuge) were placed in a Gamma Counter to measure ^{125}I content. SEM shown, n=2. Statistical analysis (t-test) indicate for a given number of washes the level of ^{125}I-Avidin binding to mAb exposed and control are not different at the 0.05 level.

3.4 Discussion

3.4.1 Characterization of [^{3}H]-nicotine binding assay

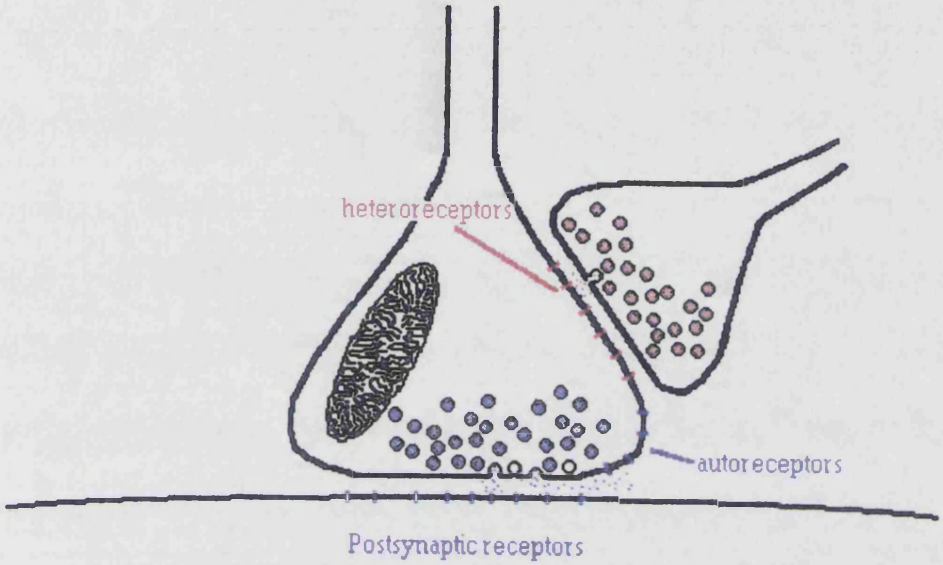
The accuracy in determining specific binding is improved by reducing the non-specific relative to specific binding (Romano and Goldstein 1980), eg by using a different buffer (Romm *et al*, 1990) as described in section 3.3.1.Ligand binding to buffer blanks (section 3.3.1),containing less than 0.5% v/v Triton, was constant demonstrating that non-specific binding is mainly due to the association of the radioligand or of radiolabelled contaminants in the radioligand (Romm *et al*, 1990) with the filter (Romano and Goldstein 1980; Schwartz *et al*, 1982). The variation in total and non-specific binding to buffer blanks where no specific binding was expected (figure 3.6) was probably attributable to random fluctuation in filtration conditions, which cannot be made more consistent, because the vacuum for each filter varies and the viscosity of the filtered solutions also varies. If the Triton concentration is high (0.5% v/v) the filtration time is over 40 sec and there is a tendency for other filters to be dried relative to the high Triton filter and ligand dissociation is increased (Lippiello and Fernandes, 1986, 1988).

The storage parameters for both detergent extracts and Tris/HEPES buffer were established (section 3.3.1). The deterioration of specific binding with buffer age probably reflected bacterial or chemical breakdown of buffer components, because freezing resolved the problem. Deterioration was not explored further, because an adequate approach had been established.

3.4.2 Analysis of synaptic junctional complexes and synaptic plasma membranes

Both SJC and the detergent solubilized (SPM-SJC) contained high affinity [^{3}H]-nicotine binding proteins in approximately equal amounts, perhaps reflecting heteroreceptors and autoreceptors respectively (figure 3.20; section 1.1). In these preliminary experiments, the protein concentration and the level of [^{3}H]-nicotine binding was low and errors were fairly high, but clearly a significant proportion of the α4ß2 nAChRs were not present in the synaptic cleft. Other evidence reinforces this conclusion. The detergent extraction procedure would remove the dense SJCs by precipitation during centrifugation (Taylor and Cotman, 1972) and this confirms that at least 28% of the α4ß2 were not present at the SJC, furthermore the distribution of SJC prepared from Percoll fractions overlaps with, but does not mirror, [^{3}H]-nicotine binding (table 3.1; section 3.4.3). The α4ß2s were not enriched in either preparation relative to P2b preparations and approximately 12% of synaptosomes have attached postsynaptic densities (eg Dunkley *et al*, 1988). It seems that α4ß2 in the synaptic cleft represent a relatively small fraction of the total α4ß2 nAChR population. It is interesting to note that Baude *et al* (1993) localized the metabotropic glutamate receptor mGluR1α using immunogold electron microscopy to a perisynaptic position, ie the receptors formed a ring around the synaptic junction. It is likely that the role of this metabotropic receptor is synaptic modulation, ie the same role proposed for the the presynaptic α4ß2 nAChR (section 1.4). However, immunolocalization of nAChRs present in the synaptic junction and cleft (SJC) might be sterically hindered by the cleft matrix, essentially as described in interpretation (b), section 5.4.3.Matrix digestion would be problematic, because the nAChRs are sensitive

Figure 3.20 Receptor locations



Presynaptic receptors may be autoreceptors which respond to neurotransmitter from the terminal itself or heteroreceptors which respond to neurotransmitter from other terminals.

Table 3.1 The distribution of protein down Percoll gradients

	A	B	C	D
F1	15.7 (2.8)	21.9 (5.4)	7.0 (1.6)	2.3 (1.8)
F2	37.3 (5.2)	33.0 (3.4)	26.3 (1.6)	12.3 (3.5)
F3	22.2 (2.6)	12.2 (3.4)	14.0 (0.8)	14.8 (2.8)
F4	5.1 (1.2)	8.0 (1.2)	8.2 (1.2)	10.3 (2.7)
F5	3.0 (1.5)	6.7 (1.3)	4.5 (0.4)	3.9 (1.7)
Recovery	83.4	81.8	35.6	--

The distribution of protein down Percoll gradients is compared to previous studies in Table 3.1, SEMs are shown in brackets. Column A shows the protein distribution in fractions from Percoll gradients described in section 3.4.3 (n=5). Column B shows the protein distribution in fractions from Percoll gradients described by Dunkley *et al*, 1988 (n=4). Column C shows the protein distribution in fractions from Percoll gradients described by Thorne *et al*, 1991 (n=6). Column D shows the proportion of synaptosomes which have attached post synaptic densities (PSDs) described by Dunkley *et al*, 1988 (n=4).

to most proteases (Whiting and Lindstrom, 1986b, c). These experiments could have been pursued, eg employing a more sensitive protein assay and performing more replicates in order to reduce the standard error of the mean, but time constraints prevented a deeper study.

3.4.3 Subcellular localisation using discontinuous density Percoll gradients

The distribution of protein between Percoll fractions of S1 prepared from rat cortex was similar to previous experiments (Table 3.1; Thorne, 1990), the differences were probably due to minor differences in preparation, or interface removal, or variations in protein estimation and [^{3}H]-ligand measurement. The variance in protein estimation and ligand binding are large in this and the previous studies (protein: Table 3.1; Thorne, 1990. Ligand: Barnes *et al*, 1992; Thorne *et al*, 1991). The degree of extraction seemed constant (about 28% protein recovery) for all Percoll fractions.

When the filtration assay was performed on fractions without detergent extraction or extensive washing, the recovery of specific [^{3}H]-cytisine binding was much greater than 100%. This demonstrates the specific [^{3}H]-cytisine binding to Percoll, but no meaningfully interpretation of nAChR distribution can be made, because the Percoll concentration varies in different fractions! Note, previous studies (Barnes *et al*, 1992; Thorne *et al*, 1991) report elevated levels of recovery of specific [^{3}H]-nicotine binding sites.

As anticipated (section 3.2.4), the standard error of the mean in non-specific

binding estimation using the immunoprecipitation assay (b) were larger than the low level of specific [^{3}H]-cytisine binding: thus the potential errors in specific binding measurement were large. This assay gave good estimates, compared with the filtration assay, of specific binding to fractions F2, F3 and F4 which contain high protein or high nAChR concentrations; but very poor estimates of specific binding to fractions F1 and F5, which contain low nAChR and low protein concentrations respectively. This approach does confirms that the filtration assay (c) predominantly measures nAChR which bind [^{3}H]-cytisine with high affinity and contain α4 and/or ß2 subunits (section 1.3). But, as anticipated in section 3.3.3, immunoprecipitation was inappropriate for a quantitative analysis of Percoll fractions.

Percoll fraction analysis using the filtration assay gave a picture for the subcellular distribution of specific [^{3}H]-cytisine binding sites which was consistent with previous localization data (section 1.4). The majority of specific ligand binding sites (fmol) were detected in the F2 fraction: fractions F4 and F5 represents only 23% of detectable binding sites. However, specific ligand binding (fmol/mg protein) was concentrated in fractions F4 and F5. Similar results were reported in previous studies, using different brain regions (Barnes *et al*, 1992; Thorne *et al*, 1991), which also show increasing specific [^{3}H]-nicotine (fmol/mg protein) to fractions down the Percoll gradient (F1 to F5). These results must be carefully interpreted to deduce the subcellular localization of the α4ß2 isoform. The distribution of nAChRs and synaptosomal neurotransmitter content cannot be reliably linked using this technique. The distribution of certain neurotransmitters, eg noradrenaline (Harrison *et al*, 1988) and to a lesser extent acetylcholine

(Thorne *et al*, 1991), do mirror the distribution of the nAChR: the distribution of other neurotransmitters, eg dopamine (Harrison *et al*, 1988), do not. Despite apparent correlations there is a considerable distributional overlap, furthermore, the concentration of α4ß2 is low and its dispersion unknown. It is entirely possible that this nAChR is widely dispersed, at low concentrations, over many subcellular particles, eg many synaptosomes, each with low nAChR concentration: alternatively or additionally the nAChRs may be concentrated to a very small subpopulation of subcellular particles, eg a small subpopulation of synaptosomes each with high nAChR concentration.

Similarly, analysis of each fraction indicates that moving down the gradient (F1 to F5) there is an increase in the numbers of large (Dunkley *et al*, 1988), synaptosomes, which contain mitochondria (Dunkley *et al*, 1988) and are functionally and metabolically viable (Harrison *et al*, 1988): functional synaptosomes are most enriched in fraction F4. The most probable interpretation is that ligand binding to fractions F5 and especially F4 measures the concentration of presynaptic α4ß2 nAChRs and the binding to other fractions measures the concentration of that isoform at other sites. But synaptosomes and other subcellular structures are found in all fractions from the Percoll gradient (Dunkley *et al*, 1988; Thorne *et al*, 1991). It is conceivable, though improbable, that the correlation between a high concentration of specific ligand binding sites and enrichment of functional synaptosomes in fractions F3, F4 and F5 is coincidental and does not reflect presynaptic localization. The possibility that the α4ß2 which were detected in Percoll fractions were exclusively postsynaptic and merely associated with attached postsynaptic densities was rejected for 4 reasons:-

a) the distribution of PSDs falls in F4 and F5 (table 3.1)

b) section 3.4.2demonstrates that a significant proportion of the nAChRs are not associated with the PSD

c) the distribution of postsynaptic receptors, eg muscarinic AChRs in the hippocampus (Thorne *et al*, 1991), show a completely different distribution, they are concentrated in F1 and F2 and virtually absent from F4 and F5.

d) an exclusively postsynaptic distribution would contradict the results of lesion studies and neurotransmitter release studies which demonstrated presynaptic nAChRs (section 1.4)

These results strongly indicate, but do not conclusively demonstrate, that at least 23% of α4ß2 receptors are located presynaptically, this compares favourably with previous studies (section 1.4; Wonnacott, 1987a). The presynaptic α4ß2 receptors seem to be concentrated on large terminals which survive enrichment using the Percoll gradient. These experiments could have been pursued (as section 3.4.2), eg employing a more sensitive protein assay and performing more replicates in order to reduce errors, but time constraints prevented a deeper study.

3.4.4 PAGE and dot-blot analyses

The dot blot analyses confirmed that primary probe detection was successful and specific, using the relevant secondary probes, and that non-specific recognition occurred at high secondary probe concentrations.

The concentration of the α4ß2 in each PAGE lane was 1.4 fmol or 0.34 ng (section 3.3.5.2).Although these approaches to immunoblotting can theoretically

detect less than 10pg per lane (De Blas and Cherwinski, 1983; Towbin *et al*, 1979), the mAbs are relatively poor immunoblotting probes (Lindstrom person. comm.). Successful immunoblot experiments reported by Whiting and Lindstrom (1987) used a 100-fold higher receptor concentration per lane (0.5-2.0pmol/lane), by examining immunopurified receptors with Lindstrom mAbs and ^{125}I-labelled goat anti-rat IgG. Immunoblotting experiments were not pursued further: considerable information had been obtained, but it was clear that the system used would not be sensitive enough using the Lindstrom mAbs to detect α4 and ß2 subunits directly in tissue preparations.

3.4.5 ELISA detection of immunostaining

The ELISA attempts (section 3.3.6)demonstrated that the mAbs were recognized by the peroxidase conjugated anti-rat IgG antibody probes, but there was considerable non-specific staining by the peroxidase conjugated anti-rat IgG antibody probes. No specific recognition of the nAChR in P2b preparations by the mAbs could be measured using the peroxidase probe. More sensitive approaches to nAChR detection were not pursued, because of the high level of non-specific staining. It was recognized that ELISA was not sensitive enough to be used reliably to detect nAChRs in P2b preparations, and the only viable approach remains that taken by the Lindstrom group, ie the detection of immunopurified receptors (section 1.4). Unfortunately the detection of immunopurified receptors compromises nAChR quantification (section 3.1.1) and provides a poor control for immunostaining and immunoaffinity experiments (section 3.1.2).

3.4.6 Immunostaining nAChRs in P2b preparations using mAbs and peroxidase conjugated anti-rat IgG antibodies

Figure 3.18 shows the level of 5AS oxidation per mg of protein, the oxidative power of samples rises with centrifugal washing, even if the level of 5AS oxidation was measured relative to sample volume (a constant for all samples!). The increase in oxidative power of samples with preparation washing (section 3.3.7; figure 3.18) was due to the release of oxidative enzymes by mitochondrial damage during centrifugation (Alberts *et al*, 1983). This trend was found in mAb exposed and control samples taken during the washing of preparations. Even if specific binding had occurred its detection would have hindered by the fact that neither preparation had constant oxidative power. This approach to immunostaining nAChRs was clearly unreliable.

3.4.7 Immunostaining nAChRs in P2b preparations using biotinylated mAbs and ^{125}I-Avidin

The potential sensitivity of ^{125}I-Avidin detection of nAChR bound mAbs was far greater than detection using peroxidase-anti(rat-IgG) antibody conjugates (section 3.4.6),see (d) below. This sensitivity, combined with the specificity of the Avidin-biotin reaction (appendix A.3), allowed an approach to immunostaining P2b preparations, which was very sensitive to any specific recognition of epitopes by the biotinylated monoclonal antibodies. No specific recognition of nAChRs containing the α4 or ß2 subunit subtypes was detected (section 3.3.7)and it seems inconceivable that any specific binding would not have been detected using this method:-

a) The recognition of the biotinylated mAbs by ^{125}I-Avidin had been confirmed by dot blot analysis (section 3.4.4).

b) Some of the ^{125}I-Avidin bound directly to the P2b preparations. This ^{125}I-Avidin was gradually removed by washing, indicating low affinity attachment.

c) The level of ^{125}I-Avidin binding was identical in mAb exposed and control (no mAb) tissues. When results were analyzed in pairs, such that samples ± mAb exposure were matched for each wash and for each experiment, the difference caused by mAb exposure was even less: there was no difference between samples ± mAb exposure, using the t-test at the 0.05 level. This observation indicates that immunostaining to the P2b preparation was entirely non-specific.

d) The concentration nAChRs was approximately 45 fmol/mg protein. The level of non-specific binding was in the order of 0.1 fmol of ^{125}I-Avidin per mg of protein and triplicate samples did not differ greatly (figure 3.19). The variation between measurements of ^{125}I-Avidin in parallel experiments was small. During mAb exposure, the cocktail of primary probes had been present in excess (10's of pmol mAb/mg protein), after washing the preparations had been exposed to 4 fmol of labelled Avidin per mg protein (1 fmol ^{125}I-Avidin measured 14,600 cpm).

A very similar approach to receptor recognition was reviewed by Lindstrom (1984). Detergent solubilized nAChRs were labelled using ^{125}I-tagged probes and immunoprecipitated. A plausible approach to examining receptor recognition under different Triton concentrations might be to use the mAb/^{125}I-Avidin system to immunostain nAChRs which had been detergent solubilized and then attached to Sepharose, using Rogers antisera. This approach was not taken for 4 reasons:-

a) the attachment of Rogers antisera to the M3-M4 region might cause long range

conformational changes to the receptors, altering epitope availability

b) this approach involves Triton exposure then removal and so it is inappropriate for an examination of the effects of Triton concentration upon recognition.

c) Triton X-100 is difficult to remove from nAChRs (Lindstrom, 1985)

d) Triton X-100 is readily iodinated (Lindstrom, 1985).

The ^{125}I-Avidin immunostaining experiments were performed, as controls, in parallel to immunoelectron microscopy experiments. Unlike the immunogold staining techniques, this approach to immunostaining is not vulnerable to extensive probe dissociation during centrifugation, because the ^{125}I-Avidin has low density and high binding affinity. Unfortunately, the ^{125}I-Avidin immunostaining demonstrates that none of the 4 Lindstrom mAbs recognize the nAChR in 10 mM PBS (pH 7.4), as suggested by immunoelectron microscopy (section 4) and immunoprecipitation (section 5) experiments.

Chapter 4 Immunoelectron microscopy

4.1 Introduction

When immunolocalization experiments are performed to localize neurotransmitter receptors, the usual approach involves staining tissue blocks (appendix A.1). The aim of the experiments described in section 4 was to immunostain subcellular particles (section 1.6). Synaptosome preparations were examined, because of the high concentration of high affinity [^{3}H]-nicotine binding sites (Rapier *et al*, 1990; Yoshida and Imura, 1979; section 1.4; appendix A.4) and in order to relate to immunoprecipitation experiments (section 5). This study continued previous, unpublished work, performed in our laboratory by B. Thorne, which had unfortunately been compromised by high levels of non-specific immunogold staining. Immunoelectron microscopy of synaptosomes was performed with 3 initial aims:-

i) to assess the viability of immunostaining

ii) to evaluate the subcellular distribution of nAChRs

iii) to evaluate the viability of dual staining

The results of immunoelectron microscopy (section 4) and parallel experiments (sections 3 and 5) indicated that the Lindstrom mAbs did not recognize the nAChR isoform under physiological conditions, thus the latter two aims could not be pursued. The experiments described in section 4 relate to an assessment of the viability of immunostaining and preliminary, unsuccessful attempts to detect immunostaining.

The optimal conditions for electron microscopy of synaptosomes were established in the late 1960's (review: Jones, 1975 Chapter 3), but various conditions have been used for an immunoelectron microscopy of synaptosomes (eg Lentz and Chester, 1977; Richardson *et al*, 1984) and related structures (Ratman *et al*, 1986a). Appendix A outlines the background to experimental decisions, summarizes the available methodological options, and describes the choice of initial preparative approach. Two representative approaches to immunostaining synaptosomes (P2b and Hb, see section 3.2.2) for nAChRs were evaluated and developmental variations to the basic protocols (section 4.2) performed with the aim of optimizing preparative conditions are described in section 4.3 (results):-

a) Prefixation immunostaining. Specimens containing synaptosomes were immunologically stained for the α4 and the ß2 subunit subtypes of nAChR, then prepared for examination with an electron microscope. This technique tends to produce excellent micrograph images, but it is most appropriate for freely accessible epitopes.

b) In postembedding immunostaining experiments preparations were immunostained after specimens had been prepared for electron microscopy, but prior to chemical staining, which allows specimen visualization. This technique produces micrographs of lower quality and only a fraction of epitopes are immunostained, so it is less sensitive, however it allows the detection of epitopes regardless of location, because there is immunological access to all parts of the cell.

If immunoelectron microscopy had been not been compromised by an unexpected failure of the antibodies to recognize the nAChR subtype, it could have been

expanded to help determine the functional role of the isoform. Immunoelectron microscopy could reveal the density and distribution of the isoform on subcellular particles in preparations from different brain regions and dual staining techniques could be used to examine the distribution of neurotransmitters and other receptors in relation to the $\alpha 4\beta 2$ isoform.

4.2 Methods

Prefixation staining methods

4.2.1 Immunostaining and fixation of the sample

Hb and P2b synaptosome preparations prepared from whole rat brains (each minus cerebellum) were initially suspended (section 3.2.2) in sucrose from the 0.8M/1.2M interface (pH 7.4, 4°C). Unless otherwise stated, all incubations described in this section:-

a) were performed at 4°C with chilled buffers and equivalent

b) proceeded using end-over-end rotation.

Synaptosome suspensions were diluted with three volumes of chilled PBS and, after centrifugation (23,000g, 30min, Sorval), resuspended (homogeniser) in chilled PBS which contained 5% w/v milk powder. After blocking (30 min) the preparation was washed by centrifugation with 20 volumes PBS (500g, 10 min, Sorval) and resuspension in 2ml PBS using a Gilson pipette. The synaptosome suspension was incubated (1h, room temperature) with the primary probe (Lindstrom antibodies), then washed by centrifugation (500g, 10 min). The synaptosome suspension was incubated (1h, room temperature) with the secondary probe (10nm gold particle labelled Avidin). Synaptosome fixation using the dual fixation technique (Sabatini *et al*, 1963) proceeded in 2 stages:

a) Glutaraldehyde was added to the suspension (to 1% v/v), the mixture was incubated (1h, room temperature) then diluted with 20 volumes of PBS and fixation was terminated by centrifugation (10 min, 500g).

b) The pellet was resuspended in 1 ml of 0.8% v/v osmium tetroxide in either 0.32M sucrose or 10mM PBS (pH 7.4, 4°C). Osmium fixation (1h, room temperature) was arrested by centrifugation (500g, 10 sec, microfuge).

4.2.2 Embedding the sample

The specimen pellet was treated as a tissue block. It was washed twice by adding 1ml water and, after 10 min, removing the water by aspiration. The pellet was fully dehydrated (Hayat, 1989a) using a series of 15 min washes in acetone/water mixtures of progressively lower water content (30%, 30%, 50%, 50%, 70%, 70%, 100% then 100% acetone). Embedding in epoxyresin (Glauert and Glauert, 1958) began with an overnight infusion of a 50:50 acetone:resin mix, followed by two overnight infusions with pure resin and finally the tissue was placed in fresh resin and cured (Marshall, 1980) by heating (60°C, 48 h) in an incubator.

4.2.3 Sectioning and positive staining the embedded sample

The resin block was cut to shape, with a razor, for sectioning of the sample area. A glass knife (Latta and Hartmann, 1950), prepared with an LKB knife maker, was used for sectioning specimens in an ultramicrotome (review: Hayat, 1989). Sections (0.5μm) were cut for light microscopy to check cutting quality. They were heat fixed, stained borax (1% w/v) containing toluidine blue (1% w/v), and viewed under oil immersion at 100 fold magnification.

Sections (100-110nm) were cut for electron microscopy using a fresh section of the knife, selected by interference colour (Peachey, 1958; Table 4.1), heat spread (to around 90nm) and collected on the dull side of a 300 square mesh grid (copper

Table 4.1 Estimation of section thickness by interference colour

Colour	thickness (nm)
grey	60
silver	90
gold	150
purple	190
blue	240

Consider the interactions of light with a thin section floating on water:-

a) some light will pass through the thin section,

b) some light will be reflected by the section as the light passes into it, ie at the upper (air/section) interface.

c) some light will be reflected by the section as the light passes out of it, ie at the lower (section/water) interface.

The reflected light from (b) and (c) will interfere and, as the wavelength of light and the thickness of the section determine the degree of constructive or destructive interference, the frequencies of light which can be seen will be determined by section thickness. To summarise the colour of a thin section indicates its thickness. Table 4.1 shows the relationship between colour and section thickness. Thus, an approximation of section thickness can easily be made, if the thickness must be more accurately determined then the interference pattern can be analyzed by spectroscopic measurements (Williams, 1977b).

or nickel). The mounted sections were positively stained (Reid, 1974). Briefly, each grid was incubated section-side-downwards on a drop of uranyl acetate (2% w/v, see section 2.2) for 10 min in the dark, humid atmosphere (see Weakley, 1981), then washed on 4 drops of water. Then, each grid was incubated on a drop of Reynolds lead citrate (0.24% lead citrate, section 2.2) for 10 min in a NaOH rich atmosphere (Reynolds, 1963), then washed on 4 drops of water. All grids were air dried.

Specimens were viewed under the electron microscope at 80 kV. Micrographs were taken at 2 magnifications (7.5k and 30k) onto photographic film. Negatives were projected onto, or used for contact-prints onto, photographic paper to give a final magnification of 30k.

4.2.4 Quantification of observations

Crude measures for the degree of damage to, and the abundance of, synaptosomes were used:-

a) The structures which could be characterized as synaptosomes in the analysis field were counted and divided into intact (ie outer membrane apparently not disrupted) and damaged categories. The degree of synaptosomal damage was assessed by estimating synaptosomal integrity, ie the percentage of undamaged, recognizable synaptosomes from the entire recognizable synaptosome population:-

$$\text{Intact synaptosomes, \%} = \frac{\text{number of intact} \times 100\%}{\text{number of detected synaptosomes}}$$

This actually represents an over-estimate of synaptosomal integrity and an under-estimate of the total synaptosomal damage, because it fails to include

synaptosomes which were completely destroyed during preparation.

b) Mitochondria are easily characterized and robust structures: it is unlikely that many were damaged beyond recognition during specimen treatment and so their frequency in the tissue preparations was assumed to be constant. The abundance of synaptosome populations was determined relative to mitochondria. A measure of the abundance of a synaptosomal population were calculated by dividing the number of synaptosomes by the number of mitochondria in a field.

$$\text{synaptosome abundance} = \frac{\text{number of synaptosomes}}{\text{total number of mitochondria}}$$

In this way the abundance of intact, damaged or detectable (intact + damaged) synaptosome populations could be calculated for comparison.

Note, as synaptosomal damage during preparation increases, (a) becomes an increasingly poor measure of damage, because it fails to include destroyed synaptosomes, (b) becomes unreliable when preparative conditions cause mitochondrial damage. Qualitative evaluation, by visual inspection, of synaptosomal content and damage is important as it can incorporate the abundance of synaptosomal components.

The distribution of gold particles in micrographs was analyzed. A random distribution of gold staining is modelled by the Poisson distribution:-

$$P(r)=e^{-\mu}\mu^{r}/r! \qquad (r=0,1,2,3\ldots \mu>0)$$

where μ is the mean number of gold particles in all quadrats,

P(r) is the proportion of quadrats which contain

r gold particles

Quadrats of defined area or volume are examined, generally by placing a regular grid of quadrats over a micrograph and counting the contents of each by hand, for an accurate analysis of gold distribution a large number (hundreds) of small quadrats (containing a mean of 2-5 gold particles) would be required. If sufficient quadrats from a micrograph are examined then a chi-squared goodness of fit test can be applied to see if the actual frequency of quadrats containing r gold particles matches the theoretical frequency predicted by the model. The value of chi squared is calculated using the formula:-

$$\text{chi squared} = \sum \frac{(\text{observed-expected frequency})^2}{\text{expected frequency}}$$

for all values where the expected frequency > 5. The expected frequencies of < 5 are pooled into groups, such that for each group the sum of expected frequencies > 5. The degrees of freedom in this case is equal to number of values for expected frequency, which are included in the calculation, minus 2. The t-test was used to compare the value of μ for quadrat populations under different conditions.

Gold staining is, of course, limited by the abundance of the antigen, which is examined. There was a relatively low concentration of the $\alpha 4\beta 2$ subtype in the specimen. An estimation of $\alpha 4\beta 2$ subtype abundance based upon [^{3}H]-nicotine binding studies (section 1.3) was made from:-

a) the concentration of high affinity [^{3}H]-nicotine binding sites ($\alpha 4\beta 2$),

b) the dimensions of each analysis micrograph,

c) the protein concentration of pellets.

As follows:-

a) [^{3}H]-nicotine binding experiments (section 3.2.6), performed upon P2b preparations, measured 90.3 ± 10.8 (SEM, n=15, figure 5.11) fmol/mg protein (section 5.3.4.1),this concentration compares well with previous studies (Irons *et al*, 1989; Wonnacott, 1987a).

b) Section thickness can be estimated, with suitable, if moderate (Williams, 1977b), accuracy from interference colour (Peachey, 1958). The dimensions of each analysis micrograph were 10.00 x 8.34 x 0.09 = 7.5 μm^3

c) the protein concentration of P2b pellets was measured using the Lowry procedure as 70 mg protein/ml of pellet.

On average, each analysis micrograph was expected to contain 28.5 high affinity [^{3}H]-nicotine binding sites or 14 α4ß2 receptors (Galzi *et al*, 1991a). Section 4.4.1.1discusses the interpretation of this estimate of α4ß2 receptor abundance.

When specific staining can be shown, stereological methods can be used to reconstruct three dimensional information. For a full discussion of these methods, refer to Williams (1977a, b) and Griffiths and Hoppeler (1986), note that autoradiographic methods are of relevance in this case.

Post fixation staining

4.2.5 Specimen embedding

The synaptosome preparations were resuspended in PBS (4°C, pH 7.4) as described in section 4.2.1. The suspension was washed once with PBS by centrifugation (500g, 10 min), resuspended in PBS (2ml) and transferred into 2

Eppendorf tubes. Synaptosome suspensions were fixed either by glutaraldehyde/ osmium tetroxide dual fixation (section 4.2.1) or by mixed aldehyde fixation (Karnovsky, 1965), as follows. Paraformaldehyde (10% w/v) was dissolved in PBS at 60-70°C and NaOH (1M) added (to 4% v/v) until the suspension cleared. The suspension was fixed by incubation (1h, 4°C) in PBS containing 2% w/v paraformaldehyde, 0.1% v/v glutaraldehyde. Free aldehyde groups were removed by exposure (1h, 4°C) to ammonium chloride (0.1M in PBS).

The tissue was embedded in LR White resin as described by Hayat (1989a). Briefly, the water washed specimen was only partially dehydrated by a series of 15 min washes at -15°C, in ethanol/water mixes of progressively lower water content (70%, 80%, 95% then 100% ethanol). Embedding began by infiltrating the specimen with a 7:3 mixture LR White:ethanol (30 min, -15°C), then with pure resin (24 h, -20°C). The tissue was transferred into fresh resin, placed into gelatin capsules and cured (24 h, 55°C).

4.2.6 Sectioning and staining

The grid used to collect ultramicrotome sections was sometimes coated with piloform plastic, then sometimes further coated with a layer of carbon (review: Hayat, 1989a, chapter 6). Briefly, a clean glass slide was dipped into 0.5% piloform in chloroform, removed and kept in a saturated chloroform atmosphere (30 sec) and then air dried (more than 2 min). A section of plastic was scored out using a diamond pen and floated off the slide onto a surface of water. Grids were placed dull side towards the plastic. A piece of dampened grease-proof paper was placed over the plastic and when removed the grids remained sandwiched

between plastic and paper. The plastic was coated with a 150nm (15 Å) layer of carbon under vacuum. The plastic around grids was scored and the coated grids stored in a grid holder at room temperature. Sections were mounted on the carbon layer coating the piloform covering the dull side of the grid.

Mounted sections were immunogold stained (Faulk and Taylor, 1971), essentially as described by Varndell and Polak (1984), immediately before uranyl acetate and lead citrate staining as described in section 4.2.3.2.Grids were placed section-side-downwards onto drops of PBS which contained protein block and/or immunological probes. Incubations proceeded in a humid atmosphere at room temperature. Sections were blocked for 30 minutes, incubated for 1 h on a drop of PBS containing the block and primary probe (Lindstrom antibodies), then washed on 4 drops of PBS/block. Next, sections were incubated for 45 min on a drop of PBS containing the block and secondary probe (10nm gold particle labelled goat anti-rat IgG), washed on 2 drops of PBS and washed on 2 drops of water. See results for details of the concentrations of the probes and block. NB, this technique can readily be adapted to double immunostaining procedures (Varndell and Polak, 1984)

Micrographs were made and printed as described in section 4.2.3.2.The image clarity was improved when examining sections on carbon coated grids by using a higher operating voltage (120 kV).

4.3 Results

4.3.1 Prefixation immunostaining: optimisation of conditions

Micrographs were examined at 30K, because it was difficult to see the subcellular components which were used to characterize synaptosomes or 10nm diameter gold particles at lower magnifications.

The quality of immunolocalisation results from a particular set of preparative conditions can be assessed by considering various criteria. The general effects of changes in preparative conditions are established (Appendix A). The relationship between preparative conditions and results criteria for prefixation immunostaining is summarized in Table 4.2. There are 2 important points:-

a) denaturation is not a problem and so harsh chemical treatments can be used after immunostaining,

b) mechanical disruption during centrifugation is potentially a major problem and must be minimized.

4.3.1.1 The choice of tissue suspension

The choice of synaptosome suspension was made empirically, based on the abundance of recognizable synaptosomes, because the effects of shear forces during preparation could not be predicted from previous work.

Plates 1 and 2 show micrographs of Hb and P2b respectively. The preparation differed from the protocol described in sections 4.2.1 to 4.2.4 (PBS buffer until

Table 4.2 Prefixation immunostaining:- Which treatment conditions affect immunolocalization results

	Antigen denaturation	Probe access	Probe-antigen dissociation	Probe-antigen association	Electron dense precipitates	Image quality and clarity	Resin stability	Synaptosomal damage
mechanical disruption during centrifugation	-	-	+	-	-	-	-	+
Buffer composition during immunostaining	-	-	u	+	+	+	-	+
Triton X-100 usage during immunostaining	u	-	?	+	u	u	-	+
Fixation regime	-	-	+	-	+	+	-	-
Dehydration	-	-	-	-	-	+	-	-
Resin type	-	-	-	-	-	+	+	-

Table 4.2 summarises which conditions during specimen treatment can affect immunolocalisation results, as defined by component results criteria.

Key

Symbol	**Can the treatment affect the results?**
-	no
u	unlikely
?	unknown
+	yes

fixation completion) in one respect:- the tissue was not pelleted and treated as a tissue block and it was washed after each treatment by centrifugation/ resuspension and not by aspiration.

Synaptosomes (Gray and Whittaker, 1962) were visible in both plates, characterized (Jones, 1975) as membrane bound particles of about 0.5 μm diameter containing mitochondria, synaptic vesicles and synaptic junctions. An electron dense precipitate could be seen in both plates and some synaptosomes were damaged but clearly recognizable. The Hb preparation contained a variety of neuronal structures of different sizes: the P2b preparation was more homogeneous and contained a greater proportion of synaptosomes and more structures seemed to be damaged. Qualitative (by visual inspection) and quantitative (section 4.2.4) estimates of synaptosome abundance and damage indicated (table 4.3, figure 4.1) that:

a) recognizable synaptosomes were more than twice as abundant in P2b than Hb,

b) a greater proportion of recognizable synaptosomes were damaged in the P2b preparation

c) by implication and by observation, it seemed that there was a greater proportion of synaptosomal debris in the P2b preparation.

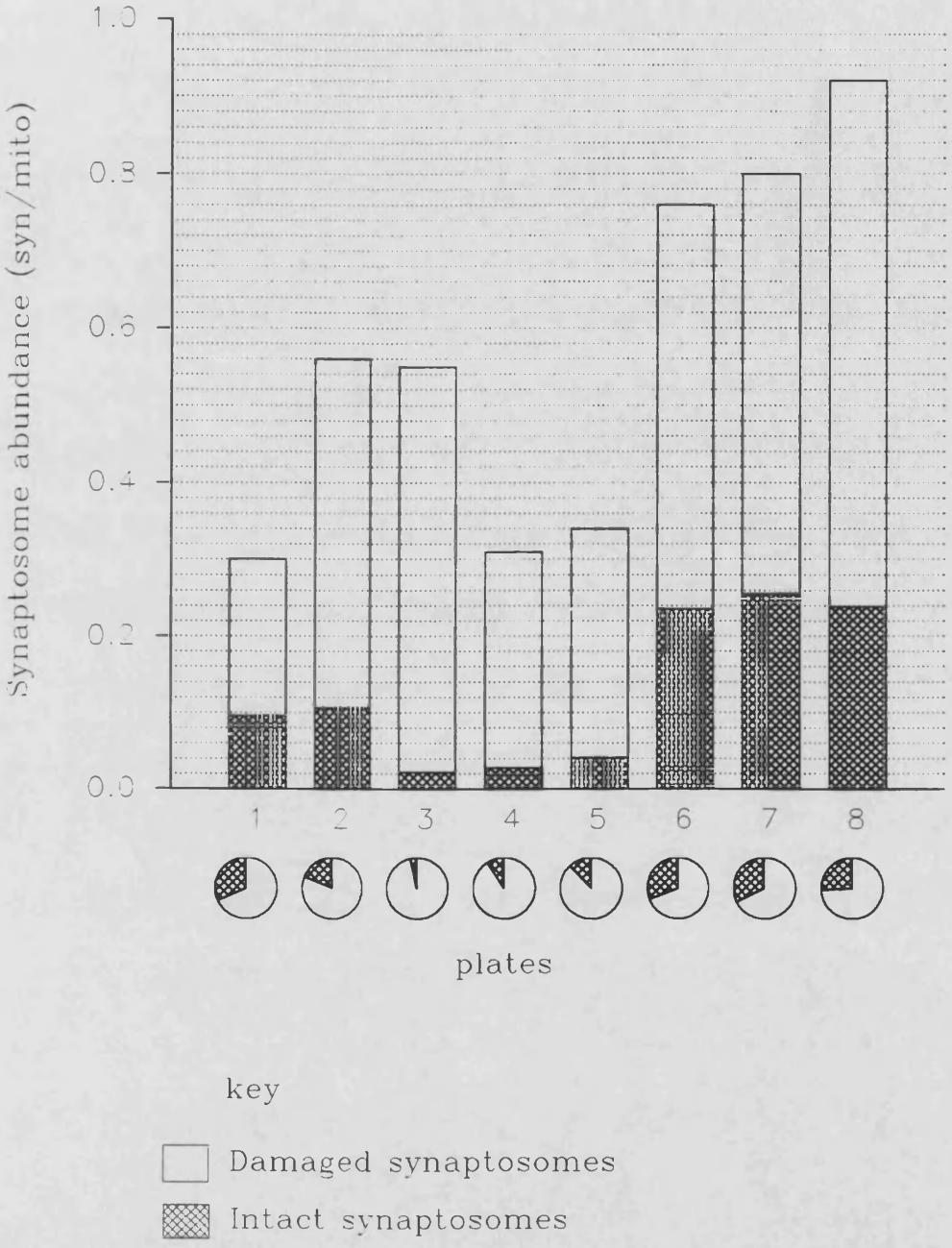
Although other structures provide a useful immunostaining control and synaptosome integrity is important, the P2b preparation was used in further experiments, because of its greater synaptosome abundance.

Table 4.3 The evaluation of synaptosome abundance and damage

plate number	1	2	3	4	5	6	7	8
Mitochondria, detectable	74	66	49	71	74	21	46	50
Synaptosomes, detectable	22	37	27	22	25	16	37	46
Synaptosomes, damaged	15	30	26	20	22	11	25	34
Synaptosomes, intact	7	7	1	2	3	5	12	12
Synaptosome abundance	.30	.56	.55	.31	.34	.76	.80	.92
Intact synaptosomes (%)	32	19	4	9	12	31	32	26

Table 4.3 displays synaptosomal integrity (intact/detected synaptosomes) and abundance (detected synaptosomes/mitochondria) for the entire analysis fields (eg plate 1a, volume 7.5 μm^3) sampled by plates 1b to 8 (see section 4.2.4).

Figure 4.1 The evaluation of synaptosome abundance and damage



The results summarized in table 4.3 are graphically displayed in figure 4.1, for intact, damaged or detectable (ie recognizable) synaptosome populations:-

synaptosome abundance = number of synaptosomes

total number of mitochondria

4.3.1.2 Consideration of mechanical damage caused by centrifugation

Mechanical disruption was an inevitable consequence of centrifugation and resuspension during synaptosome preparation and prefixation immunostaining (Fleming *et al*, 1980), previous studies demonstrated that synaptosomal damage increased with increased centrifugal washing (Dunkley *et al*, 1988; Fleming *et al*, 1980; appendix A.5). The effect of mechanical disruption upon synaptosome integrity was examined, to determine the degree of damage and to enable the evaluation of procedures to reduce damage. Plates 1, 2 and 3 represent three preparations, which required increasing amounts of washing by centrifugation: Hb, P2b and gold stained P2b respectively. The proportion of recognizable synaptosomes, which are intact falls from 32% to 4% with this increase in mechanical stress (Table 4.3, figure 4.1). Experiments were performed with the aim of reducing synaptosome damage by mechanical disruption.

a) First developmental approach to reducing mechanical damage specimen preparation: encapsulating techniques

Encapsulation techniques are often employed to reduce damage to delicate structures during specimen preparation (Glauert, 1977). Two common embedding techniques were evaluated, both were performed, as described by Glauert (1977), at the earliest opportunity, ie immediately after glutaraldehyde fixation:-

i) The Ryter and Kellenberger method of agar encapsulation. The P2b pellet was rapidly resuspended in molten agar (50°C) and the suspension rapidly cooled in

the form of a thin layer. Plates 4 and 5 show the results of embedding in agar, they differ only in the buffer used, these micrographs are best compared to plate 8. Using this technique, the abundance and integrity of synaptosomes was very low, many were damaged beyond recognition (table 4.3, figure 4.1).

ii) The Shands method of BSA encapsulation. The P2b pellet was resuspended in PBS containing BSA (10% w/v, filtered) and a drop of 25% glutaraldehyde was added to form an encapsulating matrix of cross-linked protein. Plates 6 and 7 show the results of BSA encapsulation of the P2b preparation as a pellet and a suspension respectively, they are best compared to plate 8. The proportion of intact synaptosomes and the levels of synaptosome abundance were high (table 4.3, figure 4.1). The background staining was high, some staining was of the same electron density and particle size as gold particles and some was of similar electron density and distribution to biological structures.

b) Second developmental approach to reducing mechanical damage during specimen preparation: the use of a tissue pellet

The concept of encapsulation was imitated, without any encapsulating matrix, instead a tissue pellet was treated as if it was a tissue block (Lentz and Chester, 1977). The pellet was formed at the earliest possible stage, the washing stage after the glutaraldehyde fixation. It was gently treated in the same manor as a tissue block for the rest of the specimen preparation (plate 8). When this procedure was used, micrographs show that synaptosomes were far more abundant: plate 8 has three times as many as synaptosomes as Hb, plate 1. The reason for the increase in synaptosome abundance was the decrease in synaptosomal damage and so

fewer synaptosomes were disrupted beyond recognition: 26% of recognizable synaptosomes were intact in plate 8 as compared to 19% in plate 2, (Table 4.3, figure 4.1).

c) Interpretation of developmental attempts to minimize mechanical damage and choice of protocol adjustments

Agar encapsulation (section 4.3.1.2.a.i)was an unsuccessful approach to reducing mechanical damage, probably the high temperature caused synaptosome lysis, it was not implemented. BSA encapsulation (section 4.3.1.2.a.ii) did reduce mechanical damage and provided excellent synaptosome preservation. However, it resulted in inconveniently high levels of non-specific staining and this background staining made interpretation far harder. This non-specific staining may have been the result of chemical reaction between the trapped PBS buffer and the positive stains (sections 4.3.1.3.dand 4.3.1.3.f.iii)or between the BSA itself and the positive stains. BSA encapsulation was only a partially success, only to be used if circumstances demanded, and it was not implemented.

The successful approach (section 4.3.1.2.b) reduced mechanical damage, but avoided the artifacts resulting from encapsulation. The pellets formed from this procedure were small, allowing easy penetration of acetone and resin (appendix A.10). Initially (section 4.3.1.2.b), pellets were formed after glutaraldehyde fixation (Lentz and Chester, 1977), in later experiments pellets were formed immediately after osmium fixation. Osmium tetroxide fixation caused a dramatic increase in specimen density and so a pellet can be formed very easily after this stage. The additional synaptosomal damage incurred by this change was

expected to be minimal, indeed a far lower centrifugation speed could be used after glutaraldehyde fixation, because a less dense pellet was required at that stage. Pellet formation after osmium tetroxide fixation also allowed a change of vehicle prior to osmium fixation (section 4.3.1.3.e).These protocol adjustments which minimized mechanical damage were implemented for prefixation immunostaining experiments.

4.3.1.3 Vehicle composition during synaptosome preparation, immunostaining and fixation

There is no universally appropriate vehicle for the artifact free preservation of cellular structure during fixation (Hayat, 1989a). In this particular case the selection of vehicle(s) was further complicated, as described in appendix A.6 and so an empirical evaluation of likely candidates was made.

a) Sucrose solution

Specimens prepared in unbuffered (pH adjusted to 7.4) 0.32M sucrose solution (plates 9 and 10) contain severely damaged synaptosomes. Tissue staining was good, it was better using uranyl acetate and lead citrate (plate 9) stains than lead citrate alone (plate 10). The low level of non-specific staining was unaffected by the use of uranyl acetate. This staining was a precipitation of electron dense particles which were 5 to 20 nm diameter, slightly irregular in shape and diffuse at the edges, with care it could be distinguished from gold particles.

b) Tris buffer

Specimens prepared in Tris buffered saline, TBS (plate 11) have a very high level of synaptosome damage. Staining and background precipitation was similar to that seen when unbuffered sucrose was used.

c) Cacodylate buffer

Specimens prepared in cacodylate buffer (plates 12) have good synaptosome preservation. Staining was similar to unbuffered sucrose. Background precipitation was greater than in unbuffered sucrose, but levels were unaffected by using the uranyl acetate stain.

d) Phosphate buffers

Micrographs of specimens prepared using PBS (plate 4) were superior to equimolar phosphate buffer (plate 5), but there was no difference in synaptosome preservation or levels of background staining. The level of background precipitation was much higher than from other buffers, it was no different whether (plate 2) or not (plate 13) the uranyl acetate stain was used.

e) Phosphate buffer until glutaraldehyde fixation, then resuspension in unbuffered sucrose for osmium fixation

When the PBS/sucrose procedure was used for specimen preparation (see section 4.2.1) the level of background precipitation was low (plate 3), it was between that found using cacodylate buffer (plate 12) and sucrose solution (plate 9).

NB the degree of synaptosome damage should not be considered in this case, as

the preparation (PBS/sucrose, plate 3) was washed several times, by centrifugation, during immunogold staining. The level of synaptosomal damage using the PBS/sucrose approach was very similar to the damage level found when using PBS as the vehicle throughout.

f) Interpretation of the results of varying vehicle composition

Four factors were considered in the interpretation of results:-

i) The degree of synaptosome damage

ii) The effect on synaptosome visualization

iii) The degree of non-specific staining

iv) The viability of immunological recognition

i) Synaptosomal damage

Certain vehicle components (Ca^{2+} and sucrose) tend to preserve tissue structure (Hayat, 1989a). Vehicle composition can also cause synaptosomal disruption, for example Hayat (1989a) recommends avoiding the use of Tris buffer prior to embedding. Synaptosomes immunostained and fixed using a TBS vehicle were heavily damaged (section 4.3.1.3.b), as expected. Surprisingly, synaptosomes immunostained and fixed using unbuffered sucrose were also damaged (section 4.3.1.3.a).This may be due to pH changes during fixation (Hayat, 1989a), but it is more likely that the damage reflects the increased shear forces during centrifugation, due to the higher density of the medium (appendix A.5): when synaptosomes were treated as described in section 4.3.1.3.e,ie PBS buffer then sucrose after fixation, synaptosomal damage was similar to the damage found with PBS alone, indicating the synaptosomal disruption resulted from centrifugation.

ii) Synaptosomal visualization

The choice of treatment vehicle did not affect synaptosomal visualization directly: the clarity of synaptosomal images was the same for all vehicles. Staining with lead citrate and uranyl acetate always yielded clearer synaptosomal images than staining with lead citrate alone. The choice of treatment vehicle did not affect synaptosomal visualization indirectly, for example there was no cross reaction between the vehicle and positive stains which might have necessitated the avoidance of one of the positive stains.

iii) Background staining

Uranyl acetate positive staining had no effect of the level of background staining (section 4.3.1.3) and so the precipitation was not due to chemical reactions between vehicle components (eg with PBS or cacodylate) and uranium ions (Hayat, 1989a). The electron dense precipitate found using unbuffered sucrose can only be attributed to chemical cross reaction between the glutaraldehyde and osmium tetroxide (Hopwood, 1970). In experienced hands, this osmium tetroxide/glutaraldehyde precipitation can be reduced further by careful manipulation of conditions (Hopwood, 1970). The TBS did not contribute to further precipitation, so although Tris is known to react with glutaraldehyde, this polyamine did not chemically react with the electron dense stains. Cocadylate and, to a greater extent, phosphate buffers did increase the level of background staining reflecting chemical reaction with osmium tetroxide (Hayat, 1989a). This deduction was confirmed by changing the vehicle from phosphate buffer to unbuffered sucrose prior to osmium fixation (section 4.3.1.3.e).

iv) The effect of vehicle composition upon immunostaining

Antigen recognition is affected by vehicle composition, thus the ionic buffers TBS and PBS are recommended for immunological recognition (Priestly, 1987).

g) Optimal conditions

The optimal conditions derived from the results described in section 4.3.1 were:-

a) P2b preparation was suspended in PBS,

b) the suspension was immunostained, then fixed using glutaraldehyde in PBS,

c) the suspension was transferred into unbuffered sucrose solution for osmium tetroxide fixation and then precipitated by centrifugation to form a pellet, which was treated as a tissue block for dehydration (acetone) and embedding (epoxyresin).

4.3.1.4 An initial attempt at prefixation immunostaining

a) Procedure

Plates 14 and 15 show the results of an initial attempt at prefixation immunostaining, strongly based on preliminary work by of B. Thorne (person. comm.), specimen preparation was as described in section 4.2.1, with 4 minor differences:-

a) the specimen was washed once prior to glutaraldehyde fixation and thrice prior to osmium tetroxide fixation.

b) the specimen was kept as a suspension throughout all treatments and not treated in pellet form after fixation; it was washed by centrifugation/resuspension

in the same way as the specimens seen in plates 1 and 2, section 4.3.1.1.

c) the specimen was embedded in LR White resin.

d) the primary probe was a cocktail of the Lindstrom monoclonal antibodies, the secondary probe was gold conjugated anti-rat IgG antibodies.

b) Result

The image quality is noticeably poorer than in previous micrographs, reflecting the fact that LR White resin was used (appendix A.10). No gold staining was seen in either plate.

c) Interpretation

The lack of any trace of gold staining could be ascribed to recognition failure or probe dissociation. The most likely explanation is the complete dissociation of the dense colloidal gold particle during the rather extensive centrifugation (appendix A.2, A.3). The recognition of monoclonal by secondary probe was confirmed in immunodetection (section 3) and immunoprecipitation experiments (section 5). Receptor recognition by the monoclonal antibodies could not be investigated fully until probe dissociation problems had been resolved. The optimized conditions described in section 4.3.1.3.gwere used for subsequent immunostaining attempts (section 4.3.2), because they involved far more gentle and infrequent centrifugation in order to reduce the loss of probe during washing.

4.3.2 The result of prefixation immunostaining experiments

Micrographs from specimens prepared in PBS/sucrose and treated as pellets after osmium tetroxide fixation (section 4.3.1.3.g)were judged of sufficient quality, as defined by the criteria of table 4.2,to attempt immunogold staining. The protocol described in sections 4.2.1to 4.2.4was fully implemented. Biotinylated mAbs were used as primary probes, they had been shown to recognize the nAChR under the same conditions as the unbiotinylated mAbs (section 5.3).Gold conjugated Avidin was used as a secondary probe, Avidin conjugates had been shown to recognize the biotinylated mAbs (sections 3.3 and 5.3). The Avidin-biotin system was used because of the specificity and high binding affinity of Avidin/biotin recognition (appendix A.3).

Gold particles could be seen in micrographs (plate 3), indicating the preparative procedure described in sections 4.2.1-4.2.4reduced dissociation relative to the initial attempt (section 4.3.1.4).Gold staining to P2b preparations was examined by performing 3 separate experiments, in each experiment P2b preparations were divided into portions, each representing 0.25g of original brain tissue, which were immunostained under different immunological conditions. The results of gold staining in 15 representative preparations are summarized in table 4.4a:-

a) Visual examination indicated that gold staining in all 15 preparations was randomly distributed in the regions of field where tissue was present, but largely absent in regions where tissue was absent. The tissue abundance was fairly constant in all micrographs of each prefixation immunostaining experiment, but the distribution varied.

b) The results of graphical analysis of micrographs are displayed in figure 4.2,

Table 4.4 Pre-fixation gold staining, a summary of results

Table 4.4.a

Table 4.4.a displays representative results from 3 separate experiments (column 1), where P2b preparation was immunostained under a variety of immunological conditions (columns 2 to 4). Column 3 shows the μl of biotinylated antibody cocktail (see figure 4.3), column 4 shows the μl of Avidin-gold conjugate placed in 1ml of tissue suspension. The number of gold particles in 8 quadrats, each of volume 0.94 μm^3, were recorded and the sum, mean and standard deviation of the mean of the 8 quadrats are displayed in columns 5 to 7.

a)

	Immunological conditions during staining			Number of gold particles (in 8 regions)		
Exp	Block	[1°]	[2°]	Total	mean	SEM
1	X	5	40	87	10.88	3.45
2	X	5	40	107	13.63	1.82
1	X	0	40	90	11.25	2.63
2	X	0	40	113	14.13	4.32
3	X	5	20	47	5.88	0.69
3	X	0.05	20	45	5.63	2.27
3	X	5	2	2	0.25	0.16
3	X	0.05	2	4	0.5	0.27
1	✓	5	40	36	4.5	1.10
2	✓	5	40	32	4.25	0.84
1	✓	0	40	47	5.88	2.55
2	✓	0	40	57	6.50	1.04
3	✓	5	20	27	3.38	1.27
3	✓	0.05	20	N/D	-	-
3	✓	5	2	3	0.38	0.37
3	✓	0.05	2	5	0.63	0.33

Table 4.4.b

The results of t-test analysis of gold distribution comparing each set of immunological conditions described in table 4.4.a are summarised in table 4.4b:-

vs - t-test performed between 1st and 2nd sets

\+ - blocked

\- - not blocked

both - sets pooled regardless of mAb exposure

diff - t-test shows sets differ at 0.05

not - t-test shows sets do not differ at 0.05

b)

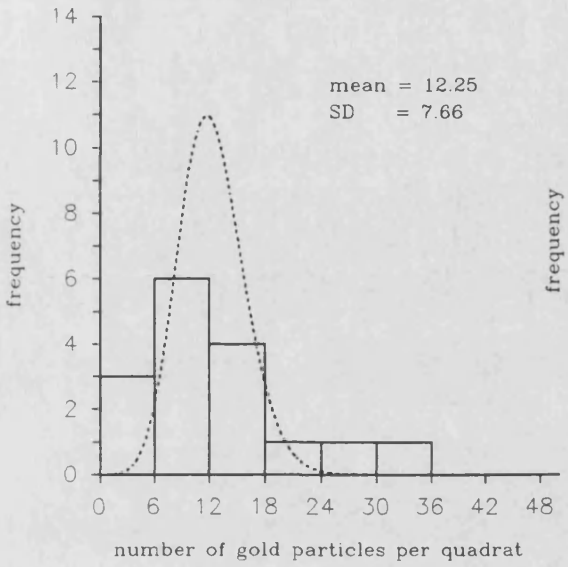
Avidin (µl)	block	mAb (µl)	diff. at 0.05 ?	t t-test	P t-test
40	-	0 vs 5	not	0.1399	0.8896
40	+	0 vs 5	not	1.217	0.2330
20	-	.05 vs 5	not	0.1055	0.9175
20	+	.05 vs 5	--	--	--
2	-	.05 vs 5	not	0.7977	0.4384
2	+	.05 vs 5	not	0.5045	0.6217
40	- vs +	both	diff.	4.199	8.7×10^{-5}
20	- vs +	both	not	1.279	0.2144
2	- vs +	both	not	0.4357	0.6661
40 vs 20	-	both	diff.	2.884	0.0060
40 vs 20	+	both	not	1.167	0.2503
40 vs 2	-	both	diff.	5.522	1.5×10^{-6}
40 vs 2	+	both	diff.	4.426	5.8×10^{-5}
20 vs 2	-	both	diff.	4.649	6.3×10^{-5}
20 vs 2	+	both	diff.	3.056	0.0058

Figure 4.2 Gold particle distribution curves

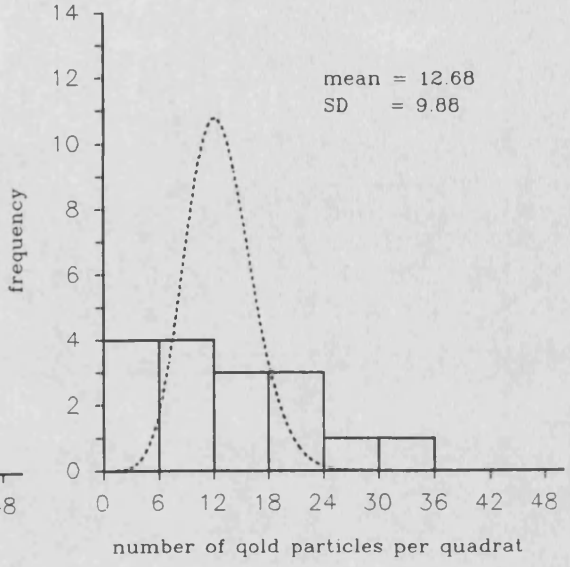
Quadrat data were primarily collected to compare immunostaining conditions: an accurate analysis of gold distribution would need a larger number of smaller quadrats. The distribution of gold particles in micrographs from experiments where blocking was not performed did not fit so well to a Poisson distribution (figures 4.2a, 4.2b, 4.2c). This was reflected in the value of chi squared (1 degree of freedom) calculated for the fit of 4.2c to its distribution, 8.1217. This fit is poor and is not acceptable at the 5% level. The distribution was skewed towards low gold distributions. On examination of this group of micrographs it was noted that the tissue density was low and uneven. The distribution of gold particles was not random, it matched the tissue distribution, ie gold particles were not washed off into regions which only contained resin. The only valid methods for avoiding this problem were to prepare a more evenly dispersed specimen or analyze at a different scale by taking more quadrats over a larger field. An invalid approach of ignoring quadrats which contain very low tissue densities produces a distribution of good fit to the Poisson distribution. As the tissue distribution was approximately equal in all of the micrographs where blocking was not performed, the conclusion that the level of staining was unaffected by mAb exposure is valid.

Despite the low quadrat numbers, the distribution of gold particles when blocking was performed matches the Poisson distribution well (figures 4.2d,4.2e,4.2f). A chi-squared goodness of fit test can only be applied at relatively high quadrat frequencies and so results of 4.2d and 4.2e were pooled to give 4.2f. The value of chi squared (1 degree of freedom) for the distribution of 4.2f to a Poisson distribution of the same mean is 0.2889. This is a very good fit which is acceptable at the 5% level.

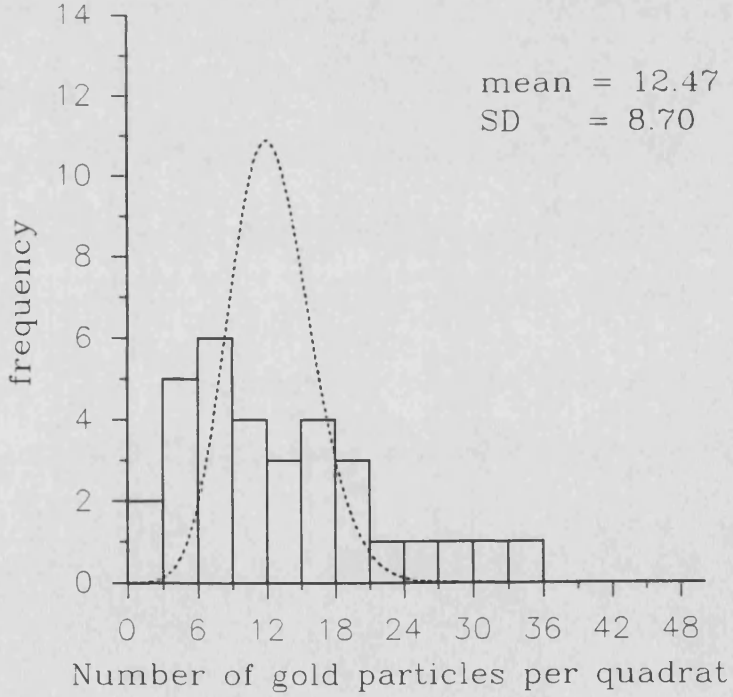
a) +mAb, no block

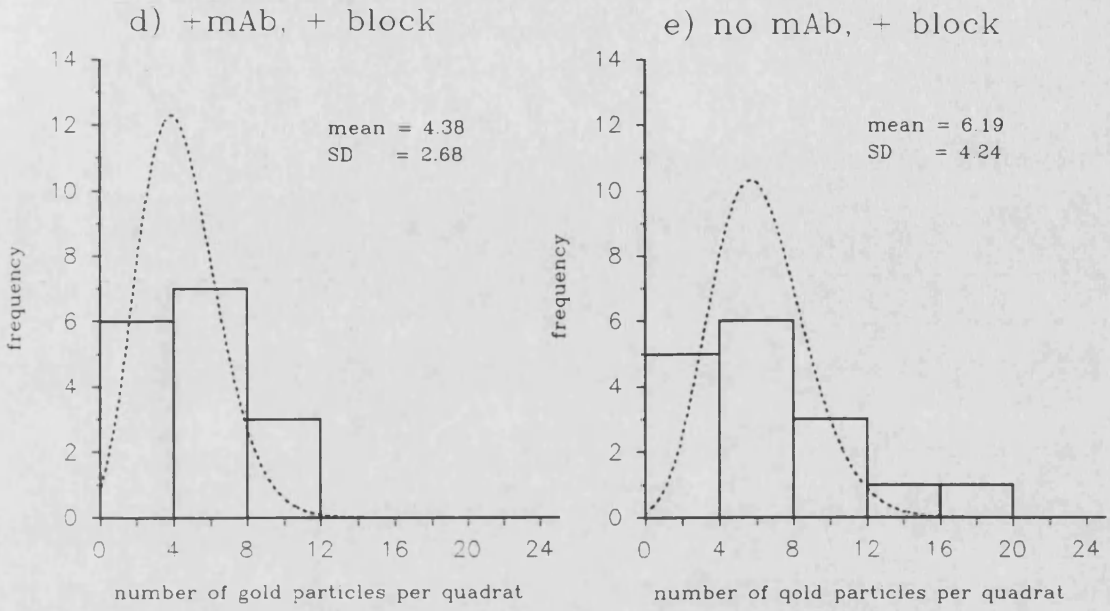


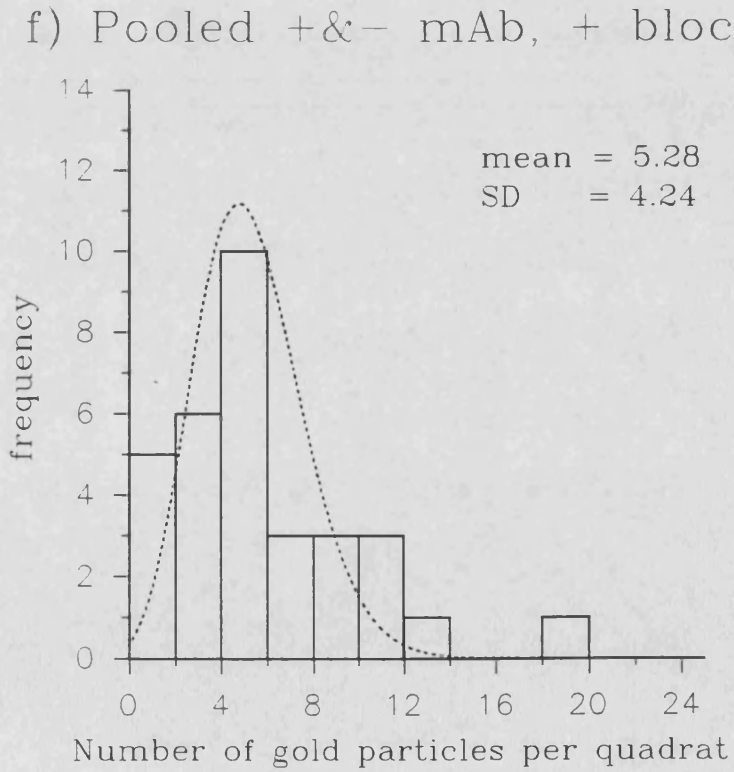
b) no mAb, no block



c) Pooled +&− mAb, no block




d) +mAb, + block
mean = 4.38
SD = 2.68
frequency
number of gold particles per quadrat
e) no mAb, + block
mean = 6.19
SD = 4.24
frequency
number of gold particles per quadrat


f) Pooled +&- mAb, + block
mean = 5.28
SD = 4.24
frequency
Number of gold particles per quadrat

they correlate with the observations described in (a). The distribution of gold particles is random and follows a Poisson distribution in regions where tissue is present. Gold staining is reduced in regions where tissue is absent, so the inclusion of quadrats covering non-tissue regions skews the distribution curves.

c) Visual inspection of micrographs indicated gold staining was entirely non-specific: compare the level and distribution of gold staining in preparations exposed (plate 3) and not exposed (plate 16) to the monoclonal antibody cocktail.

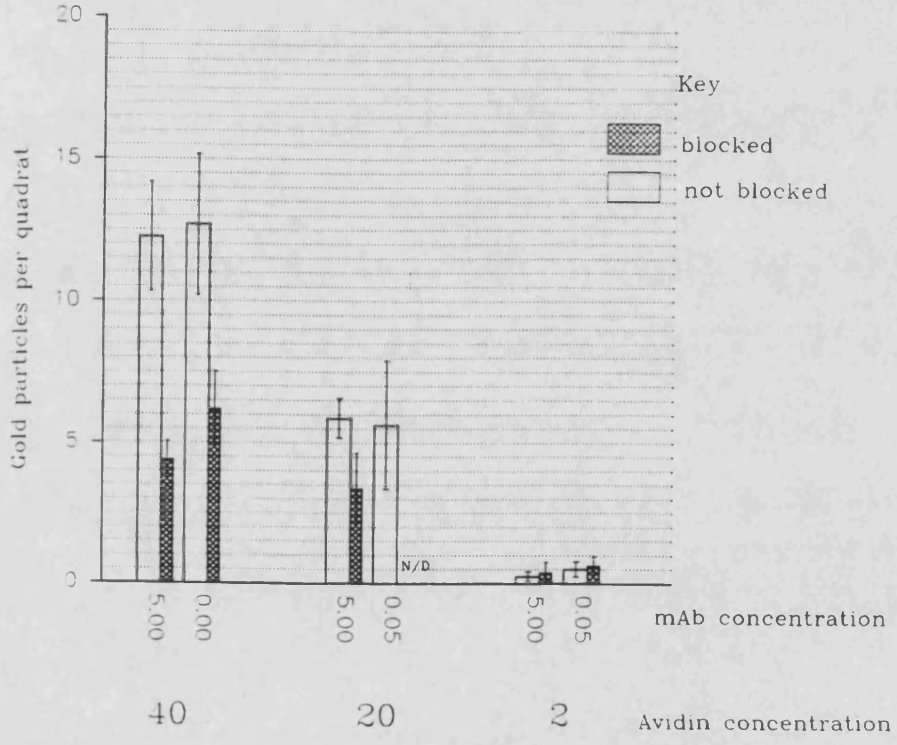
d) Statistical analysis (table 4.4b), indicates that the concentration of monoclonal antibody has no effect upon the level of gold staining (figure 4.3)

e) The degree of gold staining varied with the concentration of Avidin-gold conjugate (table 4.4b), the former appeared to be proportional to the latter (figure 4.3)

f) Blocking caused a fall in gold staining (table 4.4a). The level of gold staining was approximately halved by blocking, but remained insensitive to monoclonal concentration and directly proportional to Avidin-gold concentration figure 4.3.

No analysis was performed to correlate the distribution of gold particles with biological structures, because it had been demonstrated (above) that all detectable gold staining was non-specific.

Figure 4.3 Comparison of the effect of immunological conditions upon gold staining



The effect of altering blocking conditions, mAb concentration and Avidin-gold concentration is examined in figure 4.3.Standard error of the means (SEMs) are shown (n=8). Table 4.4b describes the results of statistical analysis of these results.

1μl of Avidin-gold contains 3.75×10^{10} gold particles

1μl of mAb cocktail in 1 ml contains 270 (40 pM), 290 (675 pM), 297 (7.5 pM), 299 (7.5 pM).

4.3.3 Optimization of post embedding immunoelectron microscopy

The second approach to immunoelectron microscopy was post-embedding immunostaining. This technique is more flexible, less sensitive and more commonly used, it also allows a general evaluation of immunostaining techniques for electron microscopy (appendix A.2).

4.3.3.1 Results of optimization experiments

The relationship between preparative conditions and results criteria for post-embedding immunostaining is summarized in table 4.5.The important points are:-

a) immunological recognition must be balanced against image quality and clarity

b) immunostaining must be balanced against the non-specific precipitation of electron dense particles

c) synaptosome damage by centrifugation can be minimized

To summarise, this immunostaining approach involves a balance in the conditions of fixation, dehydration and embedding of synaptosomes to minimize denaturation and background staining, whilst maximizing image quality.

The image quality of micrographs of specimens **embedded in LR White** (plate 14) was inferior to those embedded in epoxyresin (plate 2), but subcellular structures such as synaptosomes could still be recognized (appendix A.10). Micrographs of material fixed (section 4.2.5;appendix A.8, A.9) with **aldehydes alone** (plate 17) were of considerably lower quality than material fixed with glutaraldehyde/osmium tetroxide dual fixation (plate 14). Comparison of plates

Table 4.5 Post-embedding immunostaining:- Which treatment conditions affect immunolocalization results

	Antigen denaturation	Probe access	Probe-antigen dissociation	Probe-antigen association	Electron dense precipitates	Image quality and clarity	Resin stability	Synaptosomal damage
mechanical disruption during centrifugation	-	-	-	-	-	-	-	+
Buffer composition during immunostaining	-	-	-	+	+	+	-	-
Triton X-100 usage during immunostaining	-	-	?	?	-	-	+	-
Fixation regime	+	-	-	+	+	+	-	-
Dehydration	+	-	u	+	-	+	-	-
Resin type	+	+	u	+	-	+	+	-

This table summarises which conditions during specimen treatment can affect immunolocalisation results, as defined by component results criteria.

Key

Symbol	Can the treatment affect the results?
-	no
u	unlikely
?	unknown
+	yes

17 and 18 highlights the problem of LR White instability. Image quality and clarity was poorer and holes were rapidly burned when LR White sections were mounted without support on grids (plate 17). When sections were mounted on **coated grids** (appendix A.11) as described in section 4.2.6there was some loss of image quality and clarity and higher levels of background staining, which made the identification of biological materials more difficult (plate 18). The interpretation of micrographs was made easier by using a **higher accelerating voltage** (plate 19 and 20).

4.3.3.2 Interpretation and evaluation of optimization experiments

Sections were stabilized by mounting on support films (appendix A.11), because the LR White resin was unstable under the electron beam. The identification of synaptosomes and gold particles in film mounted sections was made harder by opaque markings resulting from variations in the coating. The coating contained atoms of low atomic mass, whilst the atoms used in positive stains (gold, uranium, lead) were of high atomic mass, so, by raising the accelerating voltage, it was possible to reduce the contrast of the markings, without affecting the biological staining.

The quality of the micrographs described in section 4.3.4 could have been improved further:-

a) In addition to marking caused by the support film staining was caused by the immunostaining vehicle. The PBS buffer used during immunostaining reacted with the positive stains to give produce a particular electron dense stain (Hayat, 1989a), which was hard to distinguish from the colloidal gold (section 4.3.4). A

more appropriate vehicle for immunostaining is TBS, which is appropriate for immunological recognition (section 4.3.1.3.f.iv; Priestley, 1987), and reacts less with positive stains (section 4.3.1.3.f.iii; Hayat 1989a). However the background staining due to the usage of the PBS buffer was negligible in comparison with the background staining resulting from the carbon coated piloform coat.

b) A significant proportion (about ⅓ to ½) of potential synaptosomes could not be identified, because of the poor image quality. There are other fixatives and resins, which might have improved image quality with negligible denaturation increases (appendix A.8.2, A.10; Hayat 1989a).

c) The proportion of available epitopes, which are immunostained, increases with decreasing gold particle size (appendix A.3). Smaller gold particles could be used, then visualized by either high magnifications or silver enhancement of the particles (Lackie *et al* 1985; pre-embedding van den Pol, 1986; appendix A.3). Neither option is desirable: small gold particles would be difficult to detect even at higher magnifications, and silver enhancement can involve nonspecific silver deposition.

The image quality of micrographs produced using postembedding techniques was poor, as anticipated from the choice of resin and fixatives (appendix A), but it was sufficient for synaptosome and gold particle identification and thus immunostaining was evaluated.

4.3.4 The results of postembedding immunogold electron microscopy

Plates 17 to 28 display the staining of Hb specimens embedded in hot and cold cured LR White resin in the presence and absence of Triton, under various blocking conditions, with and without primary probe. Specimens were fixed using the dual aldehyde method and embedded in LR White. Micrographs were made from gold stained sections which were mounted directly onto grids or coated grids. Table 4.6 summarizes these results.

a) The quality and clarity of images were poor. The image quality was at the limit necessary to recognize the features of synaptosomes, and sometimes synaptosome like structures could not be identified. The problems of synaptosome identification would have made immunolocalization difficult,

b) There was very little difference in image quality of micrographs of specimens prepared in hot (plates 18) and cold cured (plates 20) resin, when examined under identical conditions,

c) The level of gold staining, when primary probe was not present during immunostaining, was higher to sections of cold cured (plate 20) than hot cured resin (plate 18),

d) The level of gold staining, when primary probe was present during immunostaining, was higher to sections of hot cured (plate 22) than cold cured resin (plate 21),

e) The level of gold staining was higher in all cases when primary probe was present during immunostaining, than under corresponding conditions without the primary probe (eg consider +mAb in plate 22 compared no mAb in plate 18),

f) The level of gold staining was reduced in all cases when blocking conditions

Table 4.6 The results of post-embedding immunostaining experiments

Table 4.6 summarizes the results of preliminary post-embedding experiments.

Cure type, the LR White resin was either by **H**ot or **C**old cured.

mAb, whether the Lindstrom mAb cocktail was used during immunostaining.

Triton, whether Triton X-100 was used during immunostaining.

Block, 4 types of blocking were used during immunostaining:-

A - 5% goat serum + 0.2% BSA + 5% skimmed milk

B - 5% goat serum + 0.2% BSA

C - 5% goat serum + 1% BSA

D - no protein block

Coated, whether sections were mounted upon grids coated with piloform plastic covered with a carbon layer (150nm).

Voltage, the accelerating voltage used to examine sections.

Gold, the number of gold particles in the analysis entire field (7.5 μm^3, 8 quadrats).

c/c means "could not count" ie gold particles could not be counted either because the level of background staining was obstructively high or because the section or section and coating layer had detached or become otherwise lost.

Cure type	mAb	Triton	Block	Coated grids?	Voltage (kV)	Gold
C	X	X	A	√	80	c/c
C	X	X	B	√	80	c/c
C	X	X	C	√	120	82
C	X	√	A	√	80	c/c
C	X	√	B	√	80	33
C	X	√	C	√	120	c/c
C	√	X	A	√	80	c/c
C	√	X	B	√	80	328
C	√	X	C	√	120	94
C	√	√	A	√	80	29
C	√	√	B	√	80	284
C	√	√	C	√	120	c/c
H	X	X	B	X	80	15
H	X	X	D	X	80	5
H	X	X	C	√	120	1
H	X	√	B	X	80	c/c
H	X	√	D	X	80	33
H	X	√	C	√	120	90
H	√	X	B	X	80	980
H	√	X	D	X	80	≈3600
H	√	X	C	√	120	146
H	√	√	B	X	80	1644
H	√	√	D	X	80	c/c
H	√	√	C	√	120	270

during immunostaining were increased (consider no block plates 17 and 23 compared to plates 18 and 24 respectively),

g) The effect of Triton upon gold staining was difficult to determine. Many specimens could not be viewed, because the detergent caused sections on uncoated grids to dislodge, and the coating of coated grids to fold and dislodge. In all cases Triton caused extensive background staining (plate 25). Occasionally, some regions with low background staining were found (plate 26), and in these cases the level of gold staining was slightly raised (compare plate 26 and 22),

h) An anti-neurofibillary serum (ANF 2F7F10 donated by A.Rogers) was used as primary probe for a positive control (plate 27 and 28). Unfortunately, background staining was too high to allow full quantification of gold particles, however, from analysis of the few regions of low background staining, primary probe dependent staining did seem to occur,

i) Visual inspection of micrographs indicated that gold staining appeared to be entirely non-specific. The distribution of the majority of gold particles was random and was not associated with the plasma membrane of any subcellular particles or with the contents of subcellular particles, no examples of specific staining were seen,

j) Quantitative analysis (table 4.7) of micrographs, from those specimens which were immunostained with both monoclonal antibodies and gold conjugated anti-rat IgG, was performed to determine if any detectable immunostaining was specific nAChR recognition. The analysis strongly indicated that gold staining was entirely non-specific. The proportion of gold particles associated with subcellular structures mirrors the abundance of subcellular structures in the micrographs.

Table 4.7 Quantitative analysis of postembedding gold staining

Cure (Hot/Cold)	0.5% Triton?	Block (see table 4.6)	Coated grids?	Accelerating voltage (kV)	Total gold particles in field (7.5 μm^3)	Subcellular structure abundance (%)	Gold particles over subcellular structures in total quadrat area (0.75 μm^2)	Total number of gold particles in total quadrat area (0.75 μm^2)	Proportion of gold particles associated with subcellular structures (%)
C	X	A	✓	80	c/c	-	-	-	-
C	X	B	✓	80	328	51	26	51	51
C	X	C	✓	120	94	40	1	5	20
C	✓	A	✓	80	29	-	t/l	t/l	t/l
C	✓	B	✓	80	284	49	10	22	45
C	✓	C	✓	120	c/c	-	-	-	-
H	X	B	X	80	980	37	22	59	37
H	X	D	X	80	3600	42	63	146	43
H	X	C	✓	120	146	48	6	12	50
H	✓	B	X	80	1644	48	41	85	48
H	✓	D	X	80	c/c	-	-	-	-
H	✓	C	✓	120	270	49	4	8	50

Table 4.7 examines post-embedding immunogold staining in 12 micrographs, each representing different immunostaining conditions. For each section, 20 quadrats (total area 0.75 μm^2) were quantitatively analyzed: the proportion of gold particles, which were associated with subcellular structures, was compared to the abundance of subcellular structures in quadrats. The abundance of subcellular structures (%) was calculated by measuring length of quadrats covering structures and dividing by the total quadrat length, then multiplying by 100% (NB quadrat width is the section thickness and is assumed to be a constant 90nm).

Key as table 4.6, also: t/l gold distribution too low to count in quadrats

4.4 Discussion

4.4.1 Interpretation and evaluation of pre-fixation immunostaining

The images produced in pre-fixation immunostaining experiments (section 4.3.2) had predictably good quality and clarity (section 4.3.1.3.g)Synaptosomes and gold particles could be identified and were quantified. There were 3 possible reasons for the lack of detectable specific binding:-

a) The nAChR abundance was too low to be detected

b) Probes recognized the nAChR, but dissociated during specimen treatment

c) Probes failed to recognize the nAChR

4.4.1.1 α4ß2 abundance

On average each analysis micrograph was expected to contain 28.5 high affinity [^{3}H]-nicotine binding sites or 14 α4ß2 receptors (section 4.2.4).A correspondingly low level of non-specific binding must be achieved before this approach to immunolocalization could be considered viable. The estimation of α4ß2 receptor content must be interpreted with some caution:-

a) Other nAChR subtypes containing the α4 or the ß2 subunits are probably present (section 1.2 and 1.4; Wada *et al*, 1989).

b) There is some evidence that a large proportion (90-95%) of the population of receptors containing the ß2 subunit subtype are internal in soma or dendrites (Hill *et al*, 1993; Swanson *et al*, 1987). It is not clear what proportion of internal receptors can be detected by high affinity [^{3}H]-nicotine binding of synaptosomal

preparations, nor what proportion of synaptosomal ß2-containing nAChRs are internal. As receptor synthesis and storage seems limited to the soma and dendrites (Hill *et al*, 1993; Swanson *et al*, 1987), it is reasonable to assume that internal receptor content is low (Jones, 1975) at nerve terminals, this reasoning was reinforced by immunoisolation experiments (section 5.4.3). Prefixation immunostaining, using the approach described in sections 4.2.1to 4.2.4,could not be expected to detect internal receptors.

c) The distribution of the receptors is unknown, if receptors are highly concentrated on a few nerve terminals the probability of detecting a receptor rich terminal would be low. If the receptors are more dispersed, perhaps numbering 10-30 receptors per terminal (Edwards *et al*, 1990), then detection would be far more likely in a small field.

The α4ß2 receptor subtype consists of 5 subunits (Anand *et al*, 1991; Cooper *et al*, 1991), each of which are probably α4 or ß2 subtypes (Whiting *et al*, 1987a, b; Whiting and Lindstrom, 1986c, 1987, 1988). Labelling efficiency is fairly poor using colloidal gold particles, this is generally attributed to steric hinderance and labelling efficiency falls with increasing particle volume (Hayat, 1989a, b). Gold particles tend to dissociate easily and this tendency rises with increasing particle mass (Hayat, 1989a, b).

The low abundance of the nAChR means that an extensive study would be required in order to establish its distribution, even experiments to verify receptor recognition may require a very large field. The number of neurotransmitter receptors which are found at individual terminals varies enormously, from < 30

to 1000's of receptors per terminal (see Edwards *et al*, 1990). Receptor detection alone might require a field at least 10-50 times larger than the sample field used. However, the low dissociation of even non-specific binding coupled with the fact that the non-specific binding can be dramatically reduced using standard blocking procedures indicate that this approach to immunostaining nAChRs would be viable, if successful antigen recognition was achieved.

4.4.1.2 α4ß2 detection

Pre-fixation immunostaining experiments were performed (section 4.3.2) under optimized conditions (appendix A; section 4.3.1.3.g), and so good quality micrographs were produced with manageable artifacts, and gold particles were found in all micrographs. There was no detectable additional gold staining to specimens which had been exposed to monoclonal antibodies (section 4.3.2), ie non-specific staining occurred, but no specific staining was detected. These experiments indicate that the lack of specific staining can be ascribed to low abundance and/or recognition failure, but not to probe dissociation:-

a) Gold particles were randomly distributed, but restricted to those areas where specimen tissue could been seen. The lack of gold staining in, or raised levels around, the specimen interstices implies that no gold had dissociated during centrifugation.

b) The level of gold staining was inversely related to the degree of protein blocking, demonstrating its non-specific character (Hayat, 1989b).

c) The level of gold staining mirrors the concentration of secondary probe (Table 4.4). Calculations, based on the particle concentration (3.7×10^{13} particles/ml), the protein concentration (6.25 mg protein) and the dimensions of the micrographs

(section 4.4.1.1)indicate 1μl of Avidin-gold particles would result in 3⅛ particles per micrograph. The low gold loss during specimen preparation (Table 4.8) after immunostaining under non-blocking conditions demonstrates the fact that virtually no probe dissociation occurred, despite the low affinity of the non-specific attachment, this low affinity is demonstrated by protein blocking which readily reduced staining (Table 4.8).

4.4.1.3 Controls for prefixation immunoelectron microscopy

Immunocytochemical light microscopy (Dechesne *et al*, 1990; Hill *et al*, 1993; Sargent *et al*, 1989) is often performed in parallel with immunoelectron microscopy studies, rapidly to verify antigen recognition. Synaptosomes are too small (0.5 μm diameter) to be seen using light microscopy and so variations in staining would not be seen. One approach to resolving this problem would be to immunostain tissue blocks for light microscopy (Sargent *et al*, 1989; Swanson *et al*, 1987), but a closer control was chosen:- directly to measure synaptosomal immunostaining (section 3.4.6and 3.4.7).

Probe dissociation and low receptor abundance were discarded as explanations for these immunoelectron microscopy results by performing immunostaining experiments (section 3.2.9) using ^{125}I-labelled Avidin as a probe. This light molecule is much less likely to dissociate than the heavy gold conjugated probes (Hayat 1989a, b) and the sensitivity of the assay is very high so the receptor abundance was not a problem, thus the results (section 3.3.7) demonstrate recognition failure (section 3.4.7).

Table 4.8 Analysis of the loss of Avidin-gold conjugates during specimen treatment after prefixation immunostaining

Avidin-gold (µl)	40	20	2
Gold particles per micrograph (no loss)	125	62.5	6.25
Gold particles per micrograph (no block)	99.3	46	3
Remaining gold (%) - (no block)	79	74	48
Gold particles per micrograph (block)	43	27	4
Remaining gold (%) - (block)	34	43	64

The theoretical number of gold particles, assuming no loss, and the actual number of gold particles found in micrographs ($7.5\mu m^3$) are compared in table 4.8. Very little gold is lost during treatment.

4.4.2 Interpretation and evaluation of post-embedding immunostaining

The low level of gold staining in the absence of mAb incubation may have represented non-specific association of the secondary probe to the resin (Hayat 1989b) or merely inadequate washing. Non-specific association of the secondary probe is the more likely explanation because a decrease in blocking and/or the addition of Triton was associated with an increase in gold staining.

The high level of gold staining after mAb exposure might reflect specific binding, non-specific binding or inadequate washing:-

a) Inadequate washing can be discounted, because the same conditions applied for controls (no mAb, above).

b) Specific binding was not detected by visual inspection or distribution analysis (Section 4.3.4.i,j). Specific recognition was unlikely because of the low abundance of the nAChR (section 4.4.1.1 and below), however it was possible that the nAChRs are widely dispersed, or that a nAChR rich terminal was present in the analysis field, in either case specific binding may have been possible. Visual inspection is an unreliable method, because patterns are so easily seen and gold particles sometimes clump together (over subcellular structures, resin or both), however even this approach produced very few examples of possible specific binding. Distribution analysis (Table 4.7) gave no indication that gold particles were preferentially associated with subcellular structures, ie no specific binding was detected. Figure 4.4 demonstrates that the quadrats taken for distribution analysis are representative of the entire field.

c) There was good evidence that immunogold staining was non-specific:- gold

Figure 4.4 Analysis of quadrat sampling

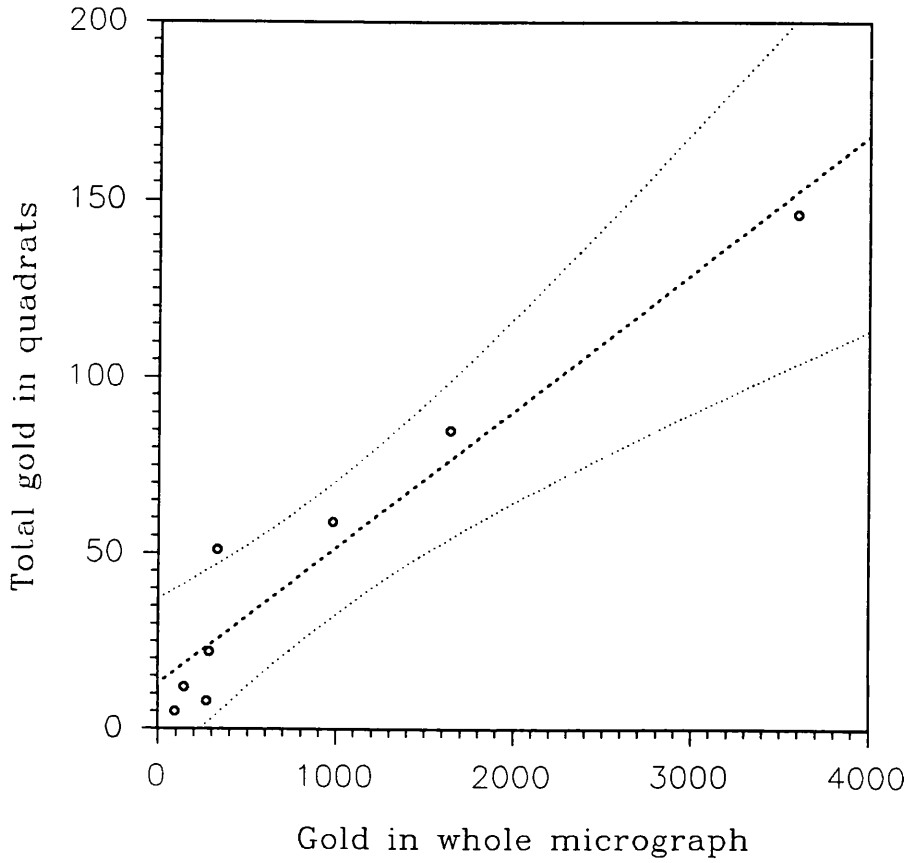


Table 4.7 examines post-embedding immunogold staining in 12 micrographs, each representing different immunostaining conditions. For each micrograph (7.5 μm^3), 20 quadrats were analyzed (total area 0.75 μm^2). Figure 4.4 demonstrates this method of sampling was appropriate, the number of gold particles in the entire quadrat area was compared with the number of particles present in the entire analysis micrograph. Linear regression indicates the two variables are proportional to each other, 95% confidence lines are also shown.

staining increased with decreased blocking and with the addition of Triton (Table 4.6; 4.7). The non-specific gold staining might have been caused by:-

i) the recognition by mAbs of non-nAChR epitopes, which were revealed during specimen treatment.

ii) the use of inappropriately high concentrations of a probe, resulted in its binding to non-specific low affinity sites (Williams, 1977a). The concentration of the secondary probe was clearly appropriate (controls). The protein concentration of the primary probe was in the range which is generally appropriate (0.7, 0.5-2.0 mg/ml Williams, 1977b), however optimal protein concentrations for probes vary dramatically.

iii) non-specific adsorption of proteins by the resin due to non-covalent interactions (Hayat, 1989a).

The level of non-specific binding might have been further reduced, by altering the concentration of the primary probe and manipulation of blocking conditions.

The total lack of detectable specific binding in postembedding experiments was almost certainly due to the low abundance of epitopes, although it is quite possible that even if receptors were present in high enough concentrations for detection, the receptor conformation would have prevented epitope recognition. Gold particles cannot penetrate the resin (Beesley, 1989; Williams, 1977a) and so specific staining is limited to recognition of antigens present in the top 10nm (approximately 10 to 16%) of the resin (Hayat, 1989b). However, the population of ß2 containing nAChRs present at the plasma membrane may (section 4.4.1.1) represent 5 to 10% of the total population (Hill *et al*, 1993; Swanson *et al*, 1987), unlike prefixation immunostaining, which would only detect plasma membrane

nAChRs, the postembedding immunostaining techniques would detect the total receptor population. This means the number of receptors which were available for recognition might be similar using both prefixation and postembedding techniques or might have been lower (approximately 10 fold) for the postembedding approach.

4.4.3 The viability of synaptosomal α4ß2 detection using immunoelectron microscopy

The results interpreted in section 4.4.1.1 indicate that immunoelectron microscopic detection of synaptosomal α4ß2 is probably viable using the prefixation approach providing a sufficiently large field is used and antigen recognition problems are resolved. A potential problem for prefixation immunostaining studies is gold particle dissociation, which can be avoided by immunostaining immobilized synaptosomes (Ratnam *et al*, 1986a; appendix A.2). However, the results interpreted in section 4.4.1.1 indicate gold particle dissociation was very low using the optimized conditions described in sections 4.3.1.3.g and 4.3.2 and immobilization procedures tend to reduce image quality and probe access, both factors are undesirable in this case.

The results interpreted in section 4.4.2 indicate that the postembedding procedure might be viable using a large field and appropriate antibodies, however this approach to nAChR localization was inferior to the prefixation technique, because it produces poorer images and higher levels of nonspecific gold staining.

To pursue this work further, 2 obstacles must be overcome:-

a) The abundance of the α4ß2 nAChRs is low, so a study of nAChR distribution using immunoelectron microscopy of synaptosomes is potentially a very large task! It is possible that the only practical approaches to immunoelectron microscopy would be either to examine immunopurified synaptosomes (Richardson *et al*, 1984), but this would omit a study of any synaptosomes which had low nAChR concentrations, or to examine tissue blocks (as Hill *et al*, 1993). In either case the original objective, of using immunoelectron microscopy of synaptosomes to examine the subcellular distribution of presynaptic nAChRs, would be compromised.

b) there is strong evidence that the Lindstrom mAbs which were used only bind to the high affinity [^{3}H]-nicotine binding nAChRs in the presence of Triton (sections 4.4.2, 3.4.7 and 5.4.3). Other antibodies are required, appendix B describes the suggested procedure for screening antibodies directed against extracellular sites of the α4ß2.

Receptor recognition problems have been encountered by other groups using the Lindstrom antibodies (Clarke, person. comm.; Wonnacott, person. comm.) and the dependence on Triton of some Lindstrom antibodies for recognition has been mentioned (Deutch *et al*, 1987; Whiting and Lindstrom, 1986a) see section 5.3 for a full discussion. Recognition problems are not limited to the Lindstrom monoclonal antibodies. Hill *et al* (1993) failed to detect the ß2 subtype at the synapse, using antibodies raised against fusion proteins, in both pre- and postembedding procedures. They proposed 2 explanations:-

a) The nAChRs containing the ß2 subunit subtype are not expressed at the synapse, instead they subserve non-synaptic neurotransmission (Umbriaco *et al*,

1991). This is a controversial explanation (section 1.4), the results of ligand binding to subcellular fractions (section 3.3.4) supports the opposing viewpoint that some $\alpha 4\beta 2$ receptor is located at presynaptic terminals.

b) The antibodies were sterically hindered from reaching nAChRs containing the $\beta 2$ subunit in the synaptic region by the subsynaptic density.

In fact, as receptor labelling was noticeably absent in Golgi saccules or vesicles, an alternative explanation related to (b) can be proposed: that the antigenic site is covered in the final stages of protein processing, ie only the internal receptor population was detected.

Recent advances in immunocytochemistry might assist immunoelectron microscopy of the nAChR:-

a) Probes can be now conjugated to 1nm diameter gold particles and visualized by silver enhancement techniques, which produce consistently shaped particles (Burry *et al*, 1993), these particles have far greater penetration, as well as the expected decrease in steric hinderance and dissociation and increase in labelling efficiency (appendix A.3).

b) Guanidine hydrochloride has been shown to reveal certain masked antigens (Peräinen *et al*, 1993).

Chapter 5 Immunoisolation experiments

5.1 Introduction

The immunoprecipitation experiments described in section 5 were designed to immunoisolate subcellular particles bearing the nAChR, and to characterize those particles (as Docherty *et al*, 1987a, b, 1989; Richardson *et al*, 1984). Monoclonal antibodies were attached to a solid matrix and used to adsorb solubilized nAChRs or nAChR bearing subcellular particles from tissue preparations. It was hoped that release studies could be performed upon immunoisolated particles (Docherty *et al*, 1987a, Richardson *et al*, 1987). The aim of these experiments was to establish more clearly the role of nAChRs by developing methods to characterize those subcellular structures where they are located.

Preliminary experiments, performed in our laboratory, were promising (Irons *et al*, 1988, 1989), but, despite several attempts, they were difficult to repeat (Wonnacott person. comm.), thus at the beginning of this investigation it was decided to verify each stage of the immunoisolation procedure. The attachment of mAbs to beads was confirmed, and then the immunoisolation of detergent solubilized nAChRs onto mAb coated beads was successfully demonstrated using each of the 4 available mAbs. However, initial attempts to immunoisolate subcellular particles (synaptosomes) bearing the nAChRs onto mAb coated beads were unsuccessful. Immunoisolation was attempted using 5 approaches in an effort to understand the situation and so develop a successful synaptosome-immunoisolation procedure:-

a) The effect of Triton was examined. Immunoisolation of nAChRs from

detergent extracts was measured at different Triton concentrations and immunoisolation of nAChRs from synaptosome preparations was measured in the presence of different concentrations of Triton.

b) Immunoisolation experiments were performed after exposure of the synaptosome preparation or the detergent extract to collagenase and exposure to collagenase with no bacterial inhibitors present. The 2 aims of this enzymic exposure were: to determine if the enzymes could interact with the nAChRs, because if the enzymes had access to the nAChRs then the mAbs should also have nAChR access; and to try to remove any proteins or parts of proteins which might mask the mAb binding sites, eg the extracellular matrix.

c) Immunoisolation experiments were performed after exposure of the synaptosome preparation to a glycosidase. The aim of this exposure was to eliminate the possibility that the mAb binding site was masked by carbohydrate residues which could easily be removed, this experiment was included for completion.

d) Some procedural variations were performed to increase the efficiency of immunoisolation, for example the synaptosome preparation was exposed to biotinylated mAbs and then exposed to Avidin coated beads, because precoating synaptosomes with mAbs often improves immunoisolation (Richardson and Luzio 1986, 1888).

e) Glutaraldehyde cross-linking was performed after exposure of the synaptosome preparation to mAbs, this procedure would tend to reinforce the weakest link - the bond between mAb and nAChR.

If immunoadsorption experiments had been successful, they could have been

expanded to help determine the functional role of the isoform. Immunopurified subcellular particles could have been characterized for neurotransmitter content, by measuring the levels of the relevant neurotransmitter synthesizing enzymes (Irons *et al*, 1988, 1989), and possibly for the presence of other receptors.

5.2 Methods

5.2.1 Tissue preparations used for immunoadsorption experiments

Sections 3.2.1and 3.2.2describe the production of Hb, P2 and P2b preparations. Detergent extracts of P2 and P2b (section 3.1.2) preparations (figure 5.1) were prepared using a method similar to that outlined in section 3.1.4. The preparations were washed once, by centrifugation, with 4 volumes of Tris/HEPES buffer (Sorval, 26,000g,30min), solubilized for 2 h in one volume of Tris/HEPES containing 0.5% v/v Triton X-100, and finally after centrifugation (Beckman, 100,000g,30 min) the supernatant was collected. In some experiments, detergent extracts of tissue preparations were not made, but instead Triton was added to the tissue preparation, which was then solubilized for 2 hours, but no centrifugal extraction was performed, then immunoadsorption and ligand binding experiments were performed upon the detergent containing tissue.

5.2.2 Immunoadsorption onto beads

Antibodies may be directly or indirectly attached to beads (Richardson and Luzio, 1986). Direct protein to bead attachment is via covalent bonding of the protein to activated beads. Indirect attachment of antibodies involves non-covalent binding to covalently attached linker proteins. Beads coated with linker proteins are sometimes commercially available, alternatively linker proteins may be attached. Unless otherwise stated in section 5.2.2:-all reactions were performed at room temperature; bead "washing"consisted of allowing them to settle, either

Figure 5.1 The detergent solubilization of P2 and P2b preparations using Triton X-100

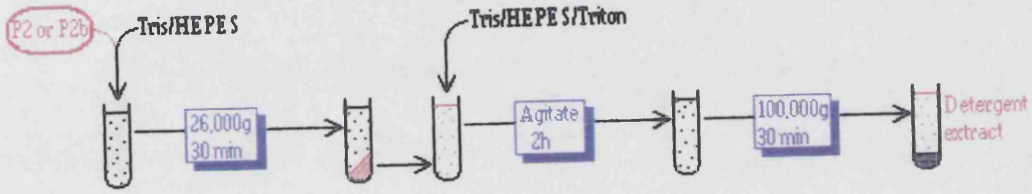


Figure 5.1 summarizes the procedure for the detergent solubilization of P2 and P2b preparations using Triton X-100.

by gravity or magnetism, removal of the old buffer by aspiration and resuspension in new buffer; all incubations proceeded under constant end-over-end rotation.

5.2.2.1 Direct attachment of proteins to cyanogen bromide activated beads

Direct protein attachment to (non-magnetic) cyanogen bromide activated beads was based on the protocol supplied. The activated gel was rehydrated (1h), without shaking, in swelling buffer (0.5% w/v, 0.1mM hydrochloric acid). Beads were washed once with coupling buffer (beads 2.5% w/v in 0.1M sodium bicarbonate, $NaHCO_3$, 0.5M sodium chloride, NaCl) and then resuspended in fresh coupling buffer (beads 5% w/v) containing the protein for attachment (protein concentration up to 0.05% w/v). After protein coupling (18 h, 4°C) beads were washed twice with two volumes of coupling buffer and resuspended in one volume of deactivating buffer (ie beads 5% w/v 1M ethanolamine, pH 8.0). After deactivation or capping of free activated sites (2h) the beads were washed for four washing cycles. Each cycle consisted of washing beads (5% w/v) in 0.1M acetic acid, 0.5M NaCl, pH 4.0, then (5% w/v) in 0.1M boric acid, 0.5M NaCl, pH 8.0. The beads were washed once (5% w/v) with, then resuspended (20% w/v) in, storage buffer (10mM PBS, 0.01% sodium azide [NaN_3], pH 7.4). Coated beads were stored at 4°C.

5.2.2.2 Direct attachment of proteins to tosylactivated beads

Direct protein attachment to (magnetic) tosylactivated Dynabeads was based on the protocol supplied. A uniform suspension of beads was mixed with an equal

volume coupling buffer (0.5M sodium borate, pH 9.5) containing the protein for attachment (0.015% w/v, protein: bead ratio 1:200). After protein coupling (24h) beads were washed several times. Each wash consisted of resuspension in two volumes of PBS/BSA (10 mM PBS, 0.1% w/v BSA, 0.01% v/v NaN_3, pH 7.4) and incubation before solvent removal. The beads were washed thrice for 10 min, then once for 30 min, and finally once (at 4°C) for 18 h. The coated beads were then stored at 4°C in fresh PBS/BSA buffer (3% w/v).

5.2.2.3 Indirect attachment of antibodies to beads

Free antibodies were indirectly attached to beads in three ways:-

a) antibodies were attached to antisera coated beads. When necessary, beads were blocked by 30 min incubation in (beads: 5% w/v) PBS containing 5% w/v Marvel milk powder, pH 7.4.

b) antibodies were attached to beads coated with immunoglobulin adsorbing proteins (Protein A or Protein G). Beads were blocked as above (a) when necessary.

c) biotinylated antibodies were attached to beads coated with biotin adsorbing proteins (Avidins). Blocking was not required.

In all cases, attachment was preceded by five washes with PBS and proceeded, either at room temperature (4h) or at 4°C (18h), in PBS (beads: 5% w/v Sepharose or 3% w/v Dynabeads) containing an excess of the anti-receptor antibodies (0.1% w/v unless otherwise stated). Unbound antibody was removed by washing the beads three times with fresh PBS (one volume, 10 min, 2% w/v).

5.2.2.4 Confirmation of antibody attachment

Both peroxidase conjugated- and alkaline phosphatase conjugated- (anti-rat) sera were used to confirm the adsorption of Lindstrom mAbs onto beads. Control beads had received identical treatment, except that mAbs were omitted from the coupling buffer. Beads were incubated (2-4 h) in PBS containing the same concentration of conjugate which was recommended for western blots (generally 0.1% v/v). A protein block (5% w/v milk powder) was not normally required. The beads were washed thrice (5 min) in PBS, before resuspension in substrate buffer.

The most visually striking substrate to use to confirm mAb attachment is one which produces an insoluble product. The insoluble product of the peroxidase catalyzed oxidation of DAB was precipitated onto beads as follows. Beads were incubated at room temperature for 10-120 sec in PBS containing hydrogen peroxide (H_2O_2, 0.6% v/v) and DAB (0.1% w/v), pH 7.4. Once the deposition was clearly visible, the reaction was stopped by washing the beads (1% v/v) in water. If the washing is not performed, even negative controls would have eventually become coated with the brown oxidation product.

Substrates which yielded soluble reaction products were used to confirm mAb attachment to beads, if the visualization of insoluble product was difficult because of bead colour, or when quantification was desired. If a peroxidase conjugated probe had been used, beads were incubated at room temperature in PBS containing 5AS, 0.2% w/v) and H_2O_2 (0.6% v/v), pH 7.4. The reaction was stopped after 30 min with 10% v/v 1M sodium hydroxide (NaOH). The soluble

product was quantified by measuring the optical density of the liquid phase at 450 nm.

When a phosphatase conjugated probe was used beads were incubated at room temperature in 50mM sodium bicarbonate [$NaHCO_3$] containing 0.1% w/v pnpp pH 9.6. The soluble product was quantified after 60 min by measuring the optical density of the liquid phase at 405 nm.

5.2.2.5 Enzyme assay

Only one enzyme assay was performed on tissue preparations, because the results of immunoisolation experiments precluded the characterization of neurotransmitter synthesizing enzymes, which may have been performed (Irons *et al*, 1988, 1989). Lactate dehydrogenase (LDH = L-lactate: NAD oxidoreductase, EC 1.1.1.27) is a cytoplasmic marker which is released upon disruption of synaptosomal membranes (Johnson and Whittaker, 1963). It catalyses the reaction:-

$$\text{Lactate} + NAD^+ \rightleftarrows \text{pyruvate} + NADH + H^+$$

LDH was directly measured by a colorimetric determination of NADH depletion (at 340nm) with its back-reaction, described by Johnson (1960). A tissue preparation which contains mitochondria is likely to contain other dehydrogenases and so reaction components were added sequentially, to ensure that the substrate dependent activity could be measured. The degree of synaptosomal integrity could

be assessed by the addition of Triton X-100, which ruptures synaptosomes and so allowing occluded LDH measurement (Marchbanks, 1967).

The substrate, co-substrate and detergent were prepared in 0.15 M Tris, pH 7.4 and the rate of change of optical density at 340nm was measured, using a spectrophotometer and recorded on a chart recorder. The rate was measured for 0.1 ml sample with 2.7ml buffer alone (0.15 M Tris, pH 7.4), and then with the addition of 0.1ml substrate (10mM sodium pyruvate [$C_3H_3O_3Na$]), and then finally with the addition of 0.1ml co-substrate (2mM ß-Nicotinamide adenine dinucleotide, reduced form [NADH]). The concentration of free LDH was calculated using the formula:-

$$\text{Activity} = \frac{A \times \text{cuvette vol} \times 1 \times 10^3}{E \times \text{vol of sample} \times p} \ \mu\text{mol.min}^{-1}.(\text{mg prot})^{-1}$$

A - change in adsorbance / min

E - Molar absorption coefficient (6.22×10^3 $M.cm^{-1}$)

l - path length (1cm)

p - protein concentration ($mg.ml^{-1}$)

The total concentration of LDH was estimated after the addition of 0.1ml detergent (10% v/v Triton X-100). The concentration of occluded LDH was calculated:-

$$LDH_{Total} = LDH_{Free} + LDH_{Occluded}$$

5.2.3 Immunoprecipitation of detergent solubilized nAChR using Lindstrom sera

Unless otherwise stated in this section all incubations were performed at 4°C, under gentle agitation using an end-over-end shaker. This method was loosely based on the protocol described by Whiting and Lindstrom (1986c). Two types of tissue were used: detergent extracts and preparations which had been exposed to the detergent, but which had not then been centrifuged to remove non-solubilized material.

Immunoprecipitation of nAChR from detergent extracts (section 5.2.1) or detergent exposed material using mAbs and beads (section 5.2.2) was performed in two ways:- either solubilized nAChR was incubated with mAb coated (directly or indirectly attached) beads for 18h; or solubilized nAChR was incubated with mAb for 18h, then the nAChR associated mAb was incubated with linker protein coated beads at 4°C (18h) or at room temperature (4h).

The immunoprecipitation of nAChR was detected in two ways:-

a) Ligand binding to beads was performed essentially as described by Flores *et al* (1992), except a different immunoadsorbant was used. Beads were dispensed into Eppendorf microfuge tubes (200μl-1000μl of 10% w/v bead suspension) and then the beads were allowed to settle by gravity or magnetically collected. The liquid removed by aspiration and the beads gently resuspended in 1ml Tris/HEPES buffer. Radiolabelled ± cold ligand were added to triplicate samples as described section 3.2.6: [^{3}H]-nicotine plus water was added to determine total binding; [^{3}H]-nicotine and (-)-nicotine were added to determine non-specific

binding. After incubating the mixtures for 30 min at room temperature, then for 18h at 4°C the bead bound nAChR was rapidly immunoprecipitated. The beads were collected either magnetically or by a centrifugation pulse in a microfuge and then the liquid was removed by aspiration. The beads in each tube were rapidly washed with 1ml of PBS. The total time for ligand removal and bead washing was less than 20 sec, it was performed at 4°C using chilled equipment and PBS. The beads were mixed (50% v/v) with 0.1 M NaOH containing sodium deoxycholate (3% w/v) and the whole transferred into a scintillation vial, containing 5ml scintillant. Tritium levels were measured with the 1600 TR.

b) Ligand binding to bead exposed detergent extract. The detergent extract was carefully removed from the beads and diluted with Tris/HEPES until the Triton concentration was 0.25% v/v. Ligand binding (section 3.2.6) to the diluted extract was compared with controls prepared from the same detergent extract, but either not exposed to beads or exposed to beads which were not mAb coated. The degree of nAChR depletion relative to controls was calculated.

5.2.4 Immunoprecipitation of material bearing nAChR

The method for the immunoprecipitation of detergent solubilized nAChR (section 5.2.3) was also used to precipitate nAChR containing tissue preparations which had not been exposed to Triton at all. The only difference in the immunoprecipitation technique was that greater care was taken when handling the beads, in an effort to avoid dissociation of nAChR bearing material from the beads. Immunoprecipitation was analyzed either by ligand binding (section 5.2.3) to, or by LDH measurement (section 5.2.2.5)of, the beads and the bead exposed tissue.

5.3 Results

5.3.1 Antibody immobilisation and detection

Table 5.1 summarizes the results of antibody immobilization by direct (section 5.2.2.1 and 5.2.2.2) and indirect (section 5.2.2.3) attachment to beads and the detection of bead bound mAbs using enzyme linked probes and [^{3}H]-nicotine binding, as described in section 5.2.2.4.The detection of mAb attachment using the phosphatase conjugated anti-(rat IgG) (0.1% v/v) was unsuccessfully attempted several times, in all cases the attachment was confirmed by peroxidase catalyzed DAB precipitation and immunoprecipitation of nAChRs. Figure 5.2 shows a representative set of results.

Antibody detection using several batches of peroxidase linked probes was successful. The peroxidase conjugated anti-(rat IgG) antibody probes were used to confirm antibody attachment using 2 types of substrate:-

a) Plate 38 shows the result of monoclonal detection by the peroxidase catalyzed deposition of the insoluble product of DAB oxidation. The brown colour of the DAB oxidation product was very similar to the colour of Dynabeads and so another method was required to confirm antibody attachment to them.

b) The soluble product of peroxidase catalyzed 5AS oxidation was measured antibody attachment. The results are summarized in 3 figures:-

i) Figure 5.3 demonstrates antibody attachment in a qualitative fashion, the attachment of all 4 Lindstrom mAbs can be confirmed in this way.

ii) Before quantification experiments were performed, steps were taken to

Table 5.1 **Confirmation of antibody immobilisation**

Matrix Linkers mAb	DAB	5AS	pnpp	nic
Seph----------290	Y	Y	N	Y
Seph----ARIgG----------270	Y	Y	N	Y
Seph----ARIgG----------290	Y	Y	N	Y
Seph----ARIgG----------297	Y	Y	N	Y
Seph----ARIgG----------299	Y	Y	N	Y
Seph----ProtA----ARIgG----all	Y	Y	n/d	Y
Seph----ProtG----ARIgG----all	Y	Y	n/d	Y
Seph----ProtA----------all	N	N	n/d	N
Seph----ProtG----------all	N	N	n/d	N
Dyn4----ARIgG----------all	n/d	Y	n/d	Y
Dyn4----Av----------all	n/d	n/d	n/d	Y
Dyn2----Av----------all	n/d	n/d	n/d	Y

Key

Seph - Sepharose 6MB

Dyn4 - Dynabeads M-450

Dyn2 - Dynabeads M-280

ARIgG - goat anti(rat IgG) antibodies

Av - Avidin

ProtG - Protein G

ProtA - Protein A

Lindstrom mAbs numbered as table 2.1 eg 270. all mAbs - all

DAB - Peroxidase linked probe, diaminobenzidine substrate

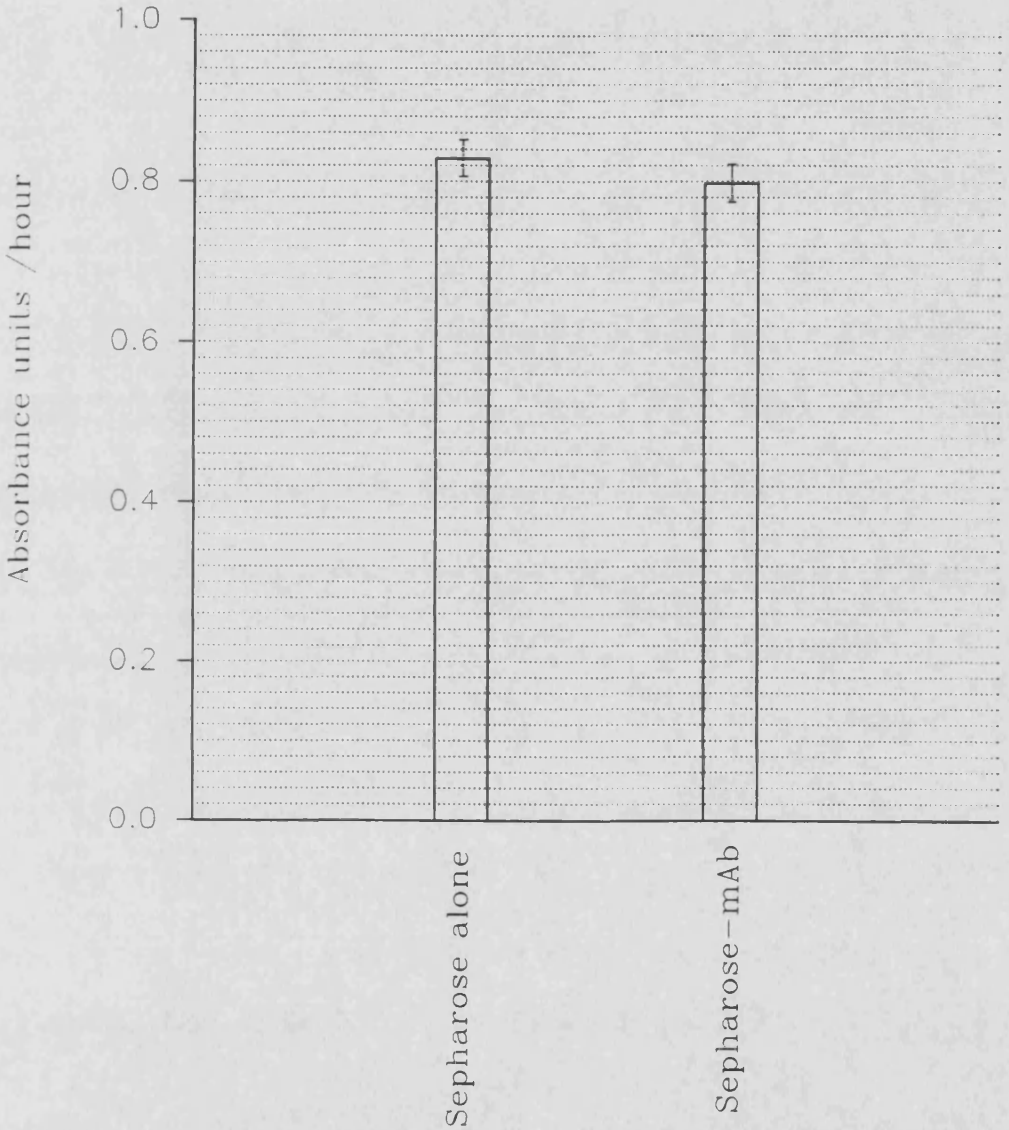
5AS - Peroxidase linked probe, 5 aminosalicylic acid substrate

Pnpp - Phosphatase linked probe, para nitrophenyl phosphate substrate

nic - [^{3}H]-nicotine binding to immunoadsorbed nAChRs.

n/d - not done

Figure 5.2 The use of a phosphatase conjugated anti-(rat IgG) probe to confirm mAb attachment



The mAb 290 was directly attached to Sepharose. The Sepharose (50μl of 5%w/v) and Sepharose-290 (50μl of 5%w/v) were protein blocked, exposed to the probe and then incubated with substrate for 1h. n=6, SEMs shown. The two populations are not significantly different at the 0.05 level (t-test, t=0.95).

Figure 5.3 The use of a peroxidase conjugated anti-(rat IgG) probe to confirm mAb attachment

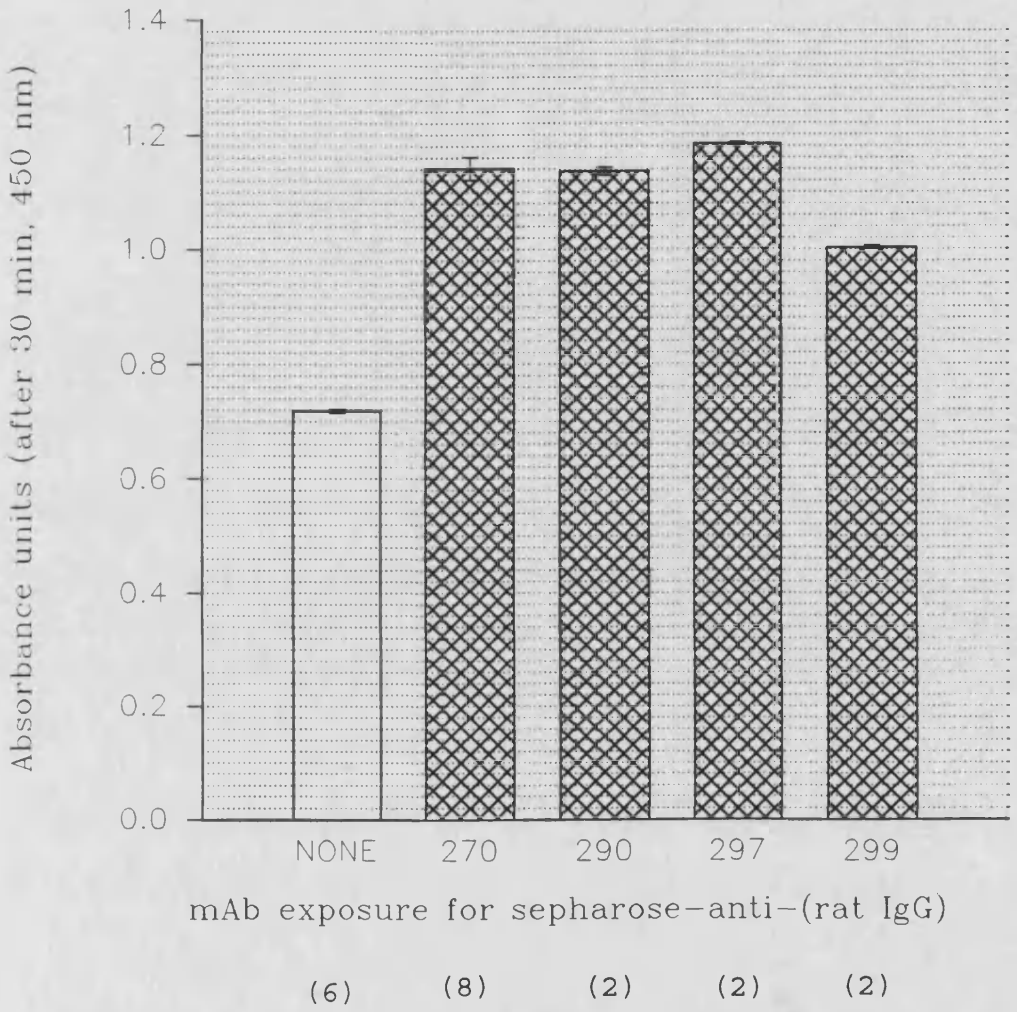


Figure 5.3 qualitatively demonstrates the detection of bead (50μl of 5%w/v) bound mAbs (0.4% v/v) by peroxidase conjugated anti-(rat IgG) (0.02% v/v). The peroxidase probe binds to each of the 4 mAbs and catalyses the oxidation of 5AS. The rate of 5AS oxidation was measured by determining the optical density (in absorbance units) at 450nm after 30 min. SEMs shown, n in brackets.

minimize 5AS oxidation, which was not caused by the presence of mAbs. It had been noticed that significant quantities of peroxidase probes tended to adsorb to the vessel wall, the selection of a vessel which did not adsorb mAbs is summarized in figure 5.4. The result was dramatic and the negligible adsorption of the secondary probe was qualitatively confirmed in later experiments.

iii) Figure 5.5 demonstrates the saturation of anti-(rat IgG) binding sites with increasing mAb concentration, when the coating of beads with mAbs is required the mAbs are used in approximately 10 fold excess to ensure complete saturation.

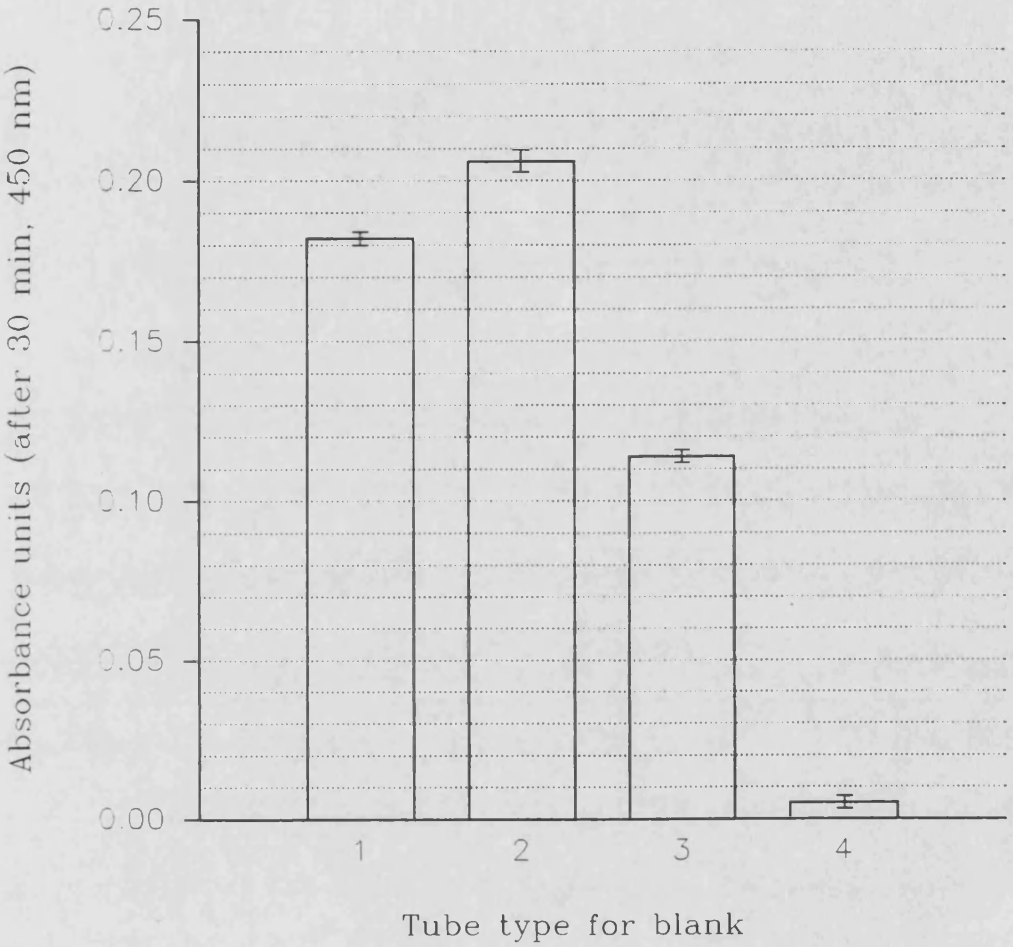
5.3.2 Immunoisolation of detergent solubilised nAChRs

The attachment of mAbs might have caused conformational changes which prevented nAChR recognition. Before adsorption attempts were made the conditions for detection were characterized and where possible optimized.

5.3.2.1 Optimization of conditions

Detergent extracts of P2 membranes were prepared as described in section 5.2.1, the recovery of protein in extracts was 28% of the protein concentration of P2 membranes (SEM=1.34%, n=5). Attempts were made to adsorb nAChRs to mAb coated beads, and to detect that adsorption. Section 3.3.1 describes optimization of the [^{3}H]-nicotine binding assay. The binding measured by filtration of [^{3}H]-nicotine to dilution series of detergent extracts of P2 membranes is summarized in figure 5.6. The level of detectable specific binding, expressed in fmol/mg protein, fell with increasing Triton concentration. Note the variance increases with decreasing Triton concentration, but this was expected (figure 5.6, section 5.4.2.1).

Figure 5.4 Reduction in non-specific 5AS oxidation by comparing the use of different vials



A single experiment (n=1) was performed to determine which vessel minimized wall adsorption of peroxidase probe (0.02%v/v) during the measurement of mAbs (0.4%v/v) adsorption onto Sepharose beads (50μl of 5%v/v). The rate of 5AS oxidation was measured (4 samples from each vessel, SEMs shown) by determining the optical density (in absorbance units) at 450nm after 30 min:-

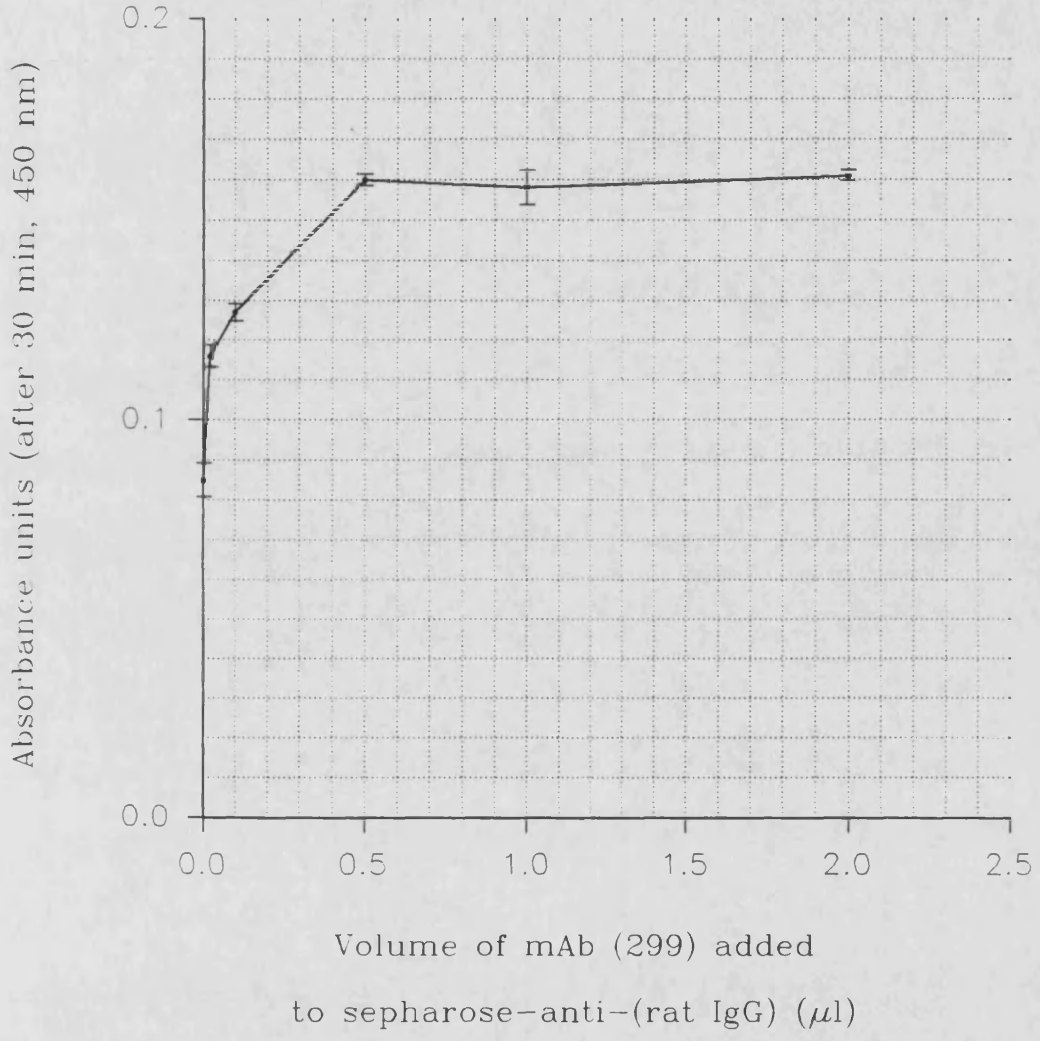
1) Lp4, white plastic tubes

2) Lp4, clear plastic tubes

3) Eppendorf microfuge tubes (1ml)

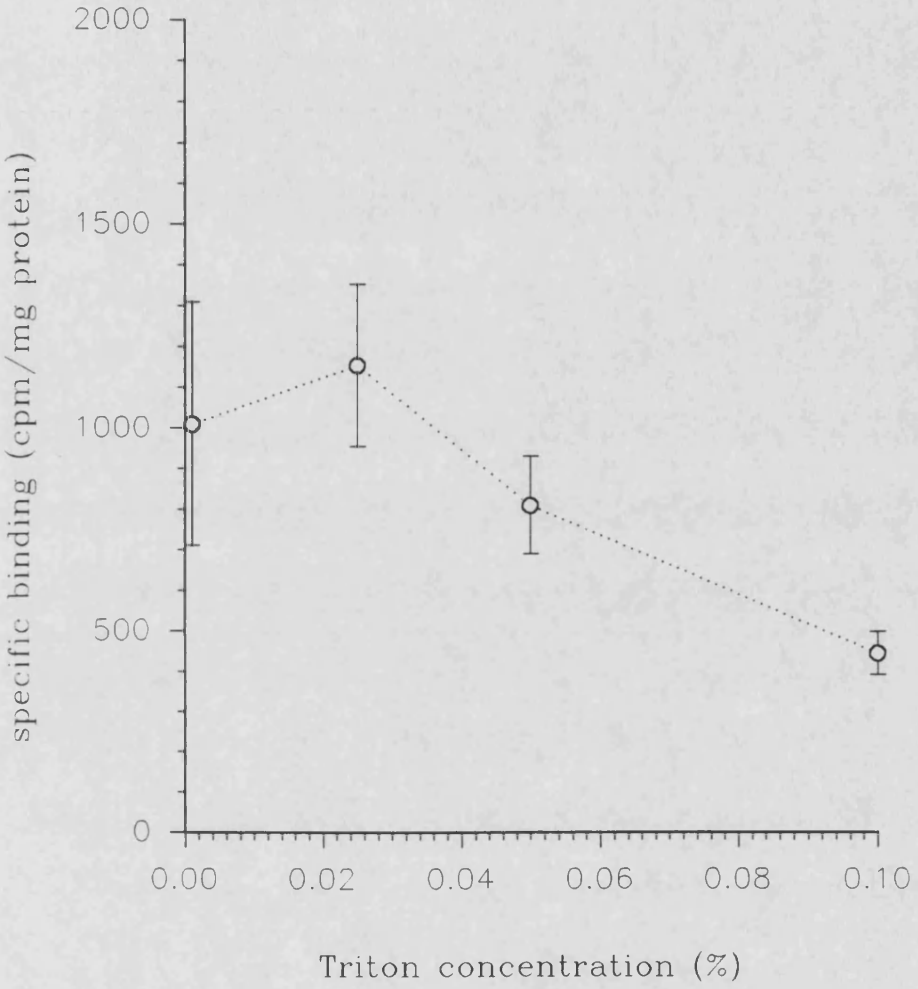
4) Glass vials

Figure 5.5 Demonstration of the saturation of anti-rat IgG sites by mAbs



The capacity of 30 μl of Sepharose--anti-(rat IgG) (5%v/v) to bind the mAb 299 is shown in figure 5.5 (n=2 for all points, SEM shown).

Figure 5.6 The effect of Triton concentration upon [^{3}H]-nicotine binding to detergent extracts of P2 membranes



Detergent extracts of P2 membranes were prepared and a dilution series made. The specific binding in cpm/mg protein verses Triton concentration is shown in figure 5.6, n=3 for all points (SEM shown). Note the protein and Triton were diluted equally and so as the Triton concentration was decreased, the protein concentration was decreased and thus the error in the binding (cpm/mg protein) was increased.

5.3.2.2 Immunoisolation of detergent solubilised nAChRs:- experimental results

The adsorption of detergent solubilized nAChR onto Lindstrom antibody coated beads (section 5.2.3) was demonstrated in two ways:-

a) the measurement (section 5.2.3.b) of the decrease in specific high affinity ligand binding to detergent solubilized preparations after bead exposure (Whiting and Lindstrom, 1986a).

b) the measurement (section 5.2.3.a)of the increase in specific high affinity ligand binding to immunoisolated nAChRs after exposure to detergent solubilized material (Whiting and Lindstrom, 1986a).

Figures 5.7 and 5.8 summarize results using these approaches

a) Each of the Lindstrom mAbs could be used successfully to deplete a detergent extract of nAChRs (figure 5.7.a), various controls (preparations 1-4) were performed to confirm that the immunoadsorption of nAChRs was only possible when mAbs were immobilized onto beads, no depletion was observed in these controls. The degree of depletion of nAChR could be increased by increasing the temperature and decreasing the vigour of agitation during immunoadsorption (preparation 6).

b) The initial approach to measuring immunoisolated nAChRs directly was to attempt acid dissociation of the receptors from the beads and then to perform a filtration assay upon the resulting solution (section 3.2.6).When this approach was tried there was no detectable [^{3}H]-nicotine binding protein, which had been dissociated from the beads (figure 5.7.b), furthermore after acid treatment there was no detectable [^{3}H]-nicotine binding to beads. The second approach to measuring immunoisolated nAChRs was to measure the binding of [^{3}H]-nicotine

Figure 5.7 The immuno-adsorption of nAChRs, detected by [^{3}H]-nicotine binding

a)

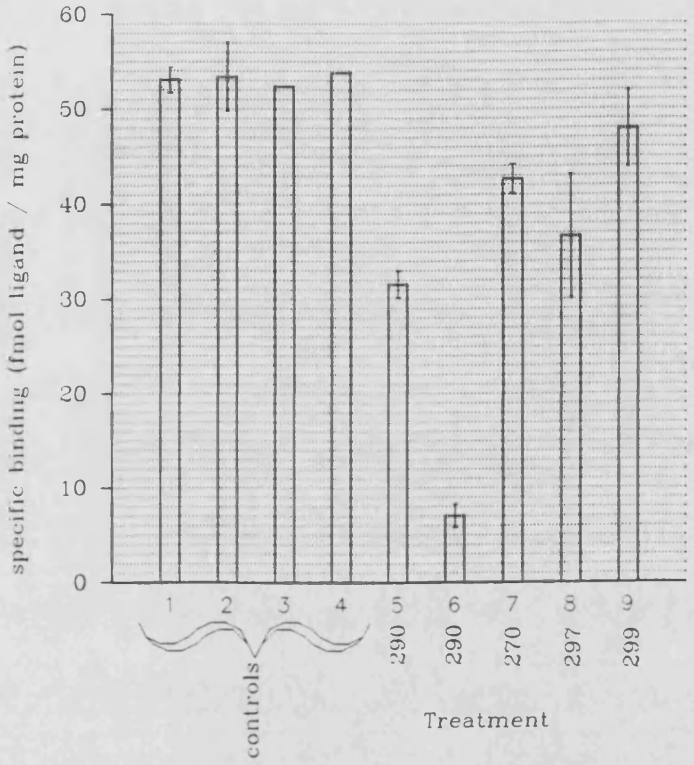


Figure 5.7.a:- nAChR immunoadsorption was performed at 0.5% v/v Triton, extracts were then diluted 10 fold, and the specific [^{3}H]-nicotine binding was measured using the filtration assay. Beads 50μl of 5%v/v. mAbs 0.1%v/v. The level of specific binding (fmol/mg protein) is displayed for:-

Treatment	n	Detergent extract exposed to
1	6	no beads
2	2	Sepharose--anti-(rat IgG), no mAb
3	1	Sepharose + mAb cocktail
4	1	Sepharose--Protein G + mAb cocktail
5	3	Sepharose--anti-(rat IgG) + 290
6	3	Sepharose--anti-(rat IgG) + 290 #2 - 18h, room temp
7	2	Sepharose--anti-(rat IgG) + 270
8	2	Sepharose--anti-(rat IgG) + 297
9	2	Sepharose--anti-(rat IgG) + 299

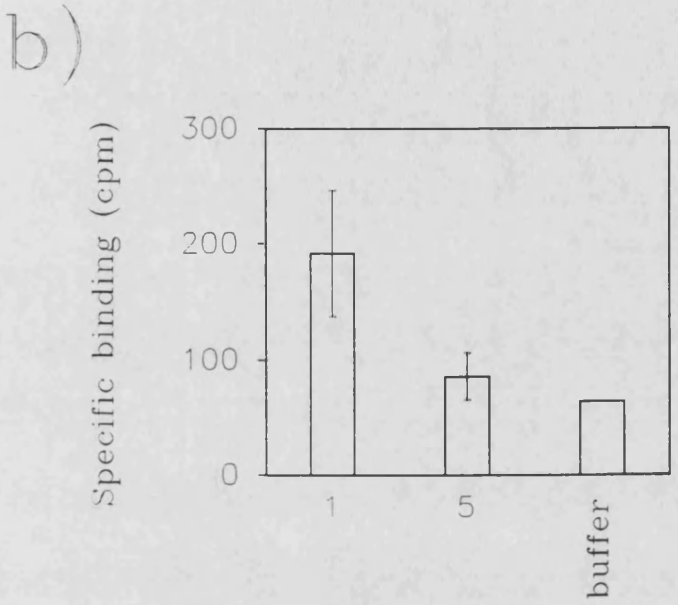


Figure 5.7.bdisplays the preliminary attempt to dissociate the nAChR from the beads by incubating them for 1h with 0.1M HCl. The acid was then mixed with 9 volumes of Tris/HEPES, the pH adjusted to 7.4 and the concentration of [^{3}H]-nicotine binding proteins in the mixture measured using the filtration assay:-

For beads from treatment (1), ie no exposure to extracts, n = 3.

For beads from treatment (5), n = 2.

For the buffer alone, ie no beads, n = 1.

Note in these experiments 100 cpm represents approximately 2.1 fmol/mg protein in the detergent extract. SEMs shown.

Figure 5.8 The detection of the transfer of nAChRs from a detergent extract of P2 membranes to mAb coated Dynabeads, the effect of Triton concentration

The transfer of nAChRs from a detergent extract of P2 membranes to mAb coated Dynabeads (50μl 3%v/v) was detected in two ways (SEM shown throughout, n shown in brackets):-

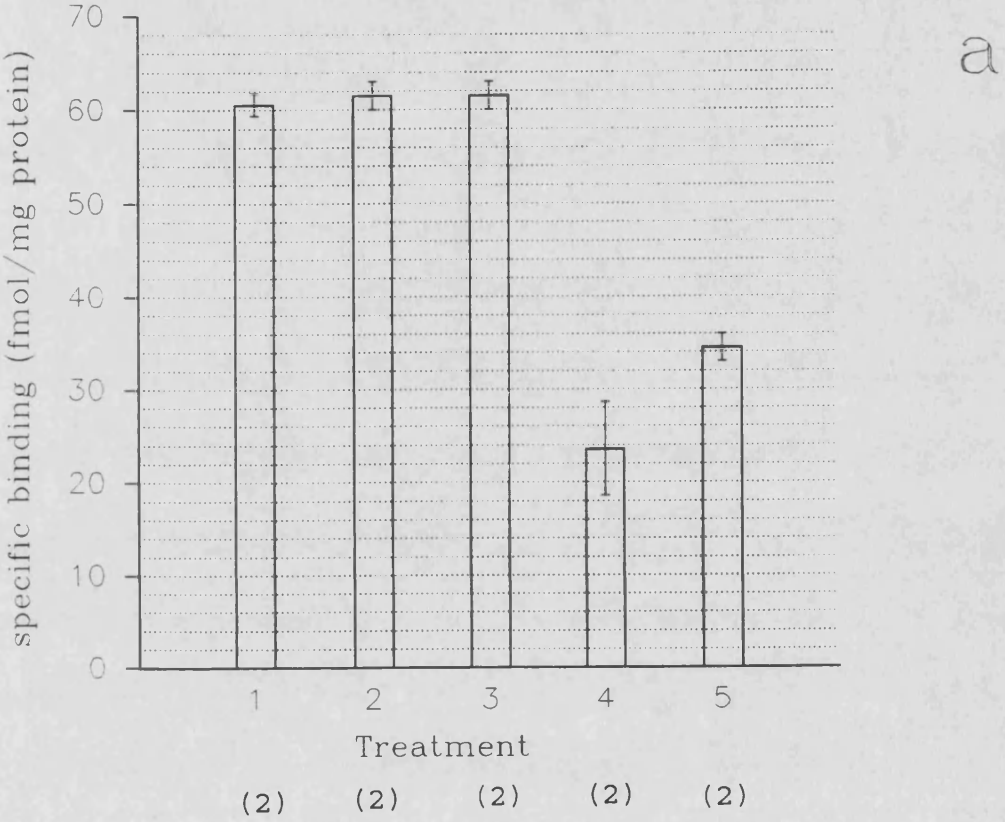
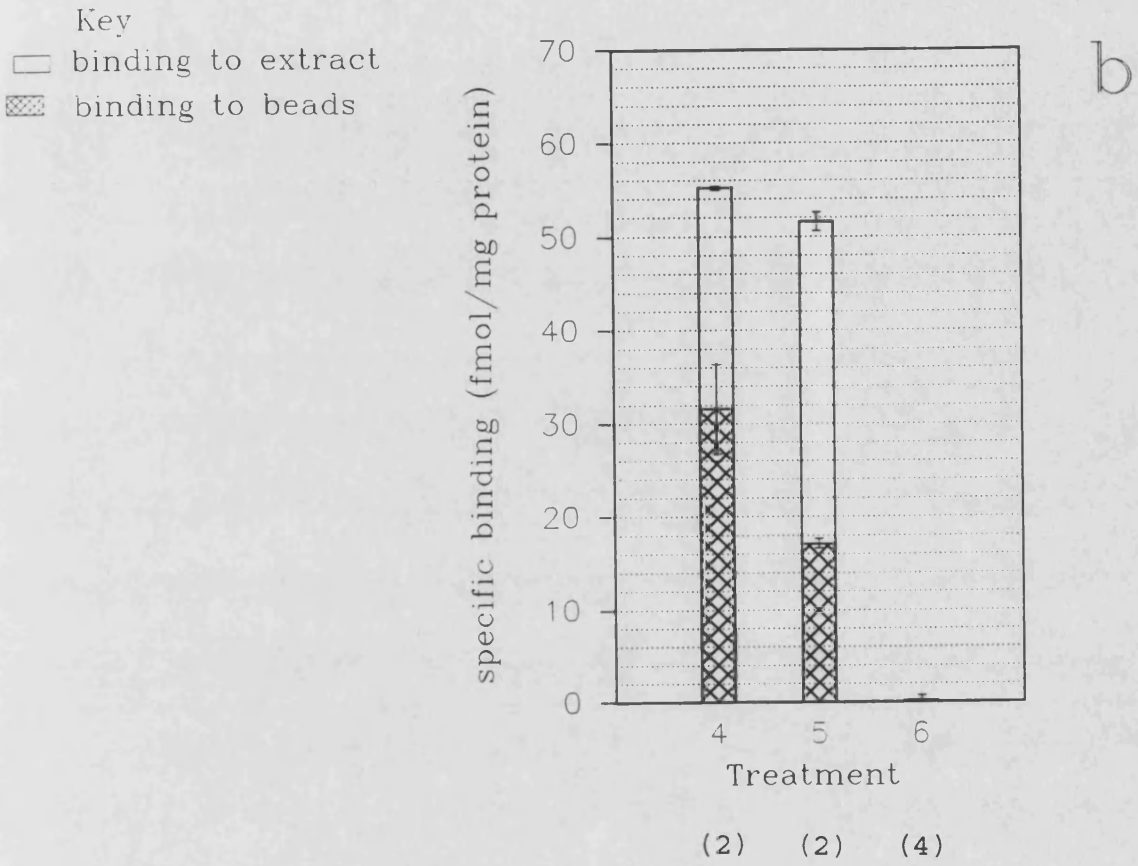


Figure 5.8.a shows specific [^{3}H]-nicotine binding to a detergent extract after exposure to beads or controls in fmol/mg protein, after compensating for the effect of Triton concentration (see figure 5.6 and figure 5.11). A detergent extract was prepared at 0.5% Triton. Part of the extract (1 vol) was diluted 10 fold with Tris/HEPES before bead exposure (treatment 4), or control (treatment 1, no beads), another part (1 vol) was diluted 10 fold with Tris/HEPES containing 0.5% Triton and then exposed to Dynabeads (treatment 5), or control (treatment 2, no beads). A control (treatment 3) was kept undiluted (0.5% Triton), then diluted to 0.05% Triton at the last minute to confirm that there was no loss of activity in a detergent extract resulting from prolonged exposure to 0.5% Triton. Binding to (4) was significantly different to combined [(1), (2) and (3)] (t-test, $t=14.2, P=7.6 \times 10^{-6}$) Binding to (5) was significantly different to combined [(1), (2) and (3)] (t-test, $t=19.2, P=1.3 \times 10^{-6}$)



b) Figure 5.8.b displays the specific binding to the beads and the mean of the sum of (bead associated specific binding plus extract associated specific binding) for both experiments. n=2. Binding to the beads was not different at the 0.05 level, but are different at the 0.1 level (t-test, t=3.0, P=0.095). Treatment 6 represents control Dynabeads (no mAbs) which were exposed to Detergent extracts containing 0.5% v/v Triton

Note when more nAChR was associated with the bead then less was associated with the extract, but the total bead + extract did not vary so much ie there was a different degree of immunoadsorption in the two experiments.

to beads as described in section 5.2.3.a. When this approach was used [^{3}H]-nicotine binding proteins associated with beads could readily be detected (figure 5.8.b), furthermore the quantity of nAChR recovered on the beads was only slightly less than that lost from the extract (figure 5.8.b).

The results displayed in figure 5.8 show two other trends:-

i) The total recovery of nAChR seemed marginally better at the higher Triton concentration

ii) The immunoadsorption of nAChR from extract to beads improved with increasing Triton concentration.

5.3.3 Immunoisolation of subcellular particles bearing nAChRs from synaptosome preparations in the absence of Triton:- initial results

The immunoadsorption of particles bearing nAChRs was monitored in two ways:-

a) detection of LDH activity

b) detection of [^{3}H]-nicotine binding proteins

a) The cytoplasmic enzyme LDH (LDH, E.C:1.1.1.27)is a cytoplasmic marker which is used to examine the degree of synaptosomal disruption:- $LDH_{occluded}$ activity reflects that synaptosome population which has not been damaged, whilst the external LDH activity (LDH_{free}) reflects damaged synaptosomes (Thorne *et al*, 1991). Figure 5.9 displays the results of LDH measurement upon fresh homogenate demonstrating that during homogenization some LDH was released

Figure 5.9 The detection LDH activity to monitor of the adsorption of subcellular particles bearing nAChRs

a)

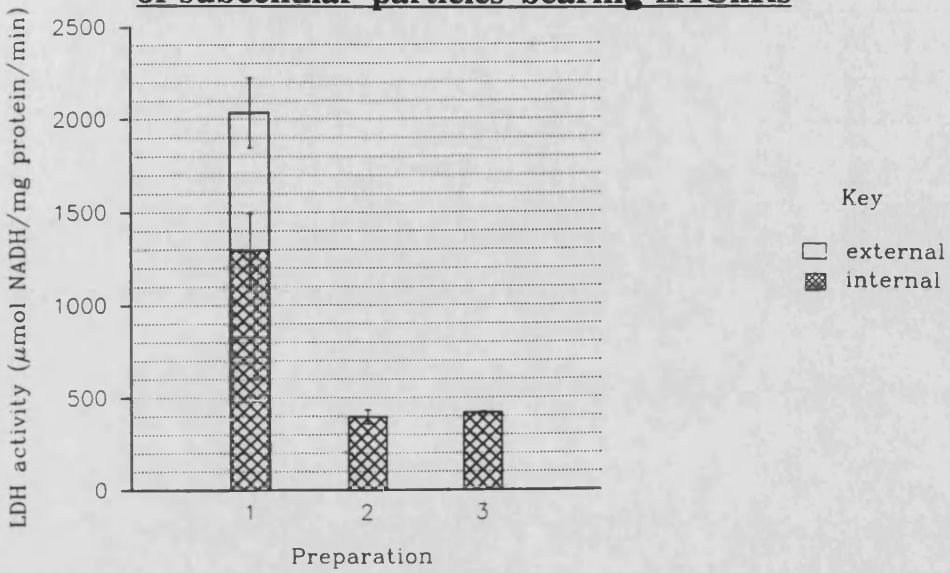


Figure 5.9.a shows the LDH activity of 3 preparations in μmol NADH produced/mg protein/min, n=3 throughout, SEM shown:-

1) Homogenate

2) Hb after exposure to control beads ie Sepharose--anti-(rat IgG) without mAbs.

3) Hb after exposure to Sepharose--anti-(rat IgG)--mAbs (50μl Sepharose, 20μl mAb cocktail).

(2) and (3) are not significantly different at the 0.05 level (t-test, t=0.593,P=0.585)

b)

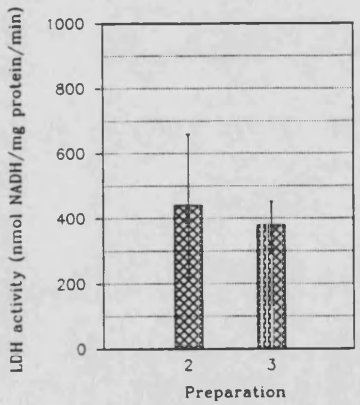


Figure 5.9.b shows the LDH activity of 3 preparations in **nmol** NADH produced/mg protein/min, n=3 throughout, SEM shown:-

2) Sepharose--anti-(rat IgG) after exposure to the Hb referred to in (2) above.

3) Sepharose--anti-(rat IgG)--mAbs after exposure to the Hb referred to in (3) above.

(2) and (3) are not significantly different at the 0.05 level (t-test, t=0.265,P=0.804)

(LDH_{free}) and some LDH remained occluded ($LDH_{occluded}$). Fresh Hb contained virtually no LDH_{free}, because it had been washed away. A single experiment was performed which indicated that occluded material was released with time: after storage for 36h at 4°C homogenate contained 29% occluded LDH and Hb contained 43% occluded LDH. In all experiments controls were performed, where the order of cosubstrate and Triton addition was altered, in all cases (± Triton) no enzyme activity was detected until both cosubstrates had been added. There was no depletion of LDH_{total} or $LDH_{occluded}$ activity during exposure to mAb coated beads. There was very little activity associated with the beads after attempted immunoadsorption: the total LDH activity associated with the beads was approximately 1000 fold less than with the $LDH_{occluded}$ activity present in Hb preparations and there was no difference in LDH activity associated with control beads than with mAb coated beads.

b) It was possible that immunoadsorption had occurred at level too low for detection with this assay and so the more sensitive [^{3}H]-nicotine binding experiments were performed to investigate the distribution of nAChRs. The immunoadsorption of nAChRs from P2b preparations in the absence of Triton was monitored by measuring [^{3}H]-nicotine binding. Figure 5.10 displays the results of immunoadsorption experiments, neither depletion of nAChR from P2b preparations nor adsorption of nAChR to the beads occurred. Note the variance in specific binding to P2b preparations (figure 5.10.c) reflected vacuum problems which occurred during this series of experiments: two pumps were used, one of which produced an inconsistent vacuum. Whilst there was considerable variation between experiments, results were relatively consistent within experiments, thus

Figure 5.10 The use of [^{3}H]-nicotine binding to monitor the immunoadsorption of nAChRs from P2b preparations in the absence of Triton

Throughout the experiments summarized in figures 5.10 [^{3}H]-nicotine binding to preparations was performed using the filtration assay, and [^{3}H]-nicotine binding to beads was measured directly as described in section 5.2.3.SEMs are displayed and n values are shown in brackets. The numbers on the x-axis of figures 5.10.a,b and c refer to the following:-

1) P2b preparation after exposure to control beads ie Sepharose--anti-(rat IgG) without mAbs.

2) Sepharose--anti-(rat IgG) after exposure to the P2b preparation referred to in (1) above.

3) P2b preparation after exposure to Sepharose--anti-(rat IgG)--mAbs (50μl Sepharose, 20μl mAb cocktail).

4) Sepharose--anti-(rat IgG)--mAbs after exposure to the Hb referred to in (3) above.

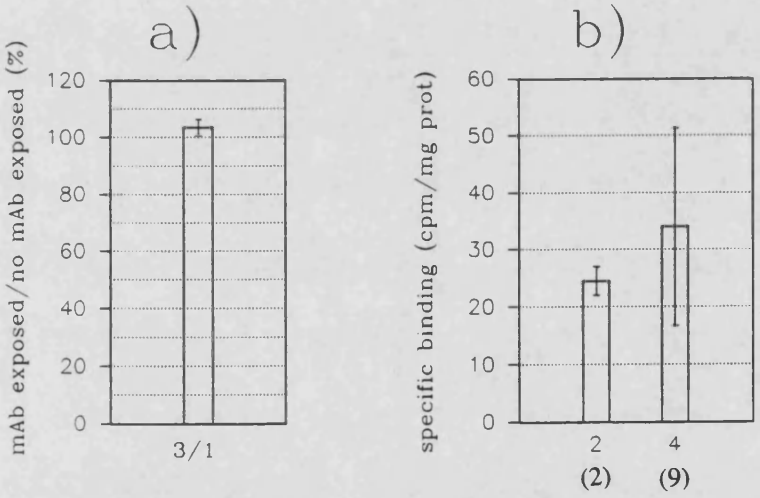


Figure 5.10.acompares the P2b preparations which have and have not been exposed to mAb coated Sepharose, ie for each experiment:- P2b preparation (3) x 100% / P2b preparation (1)
Note this comparison compensates for variation between experiments, which was considerable.
n = 14, mean = 103.5, SEM = 2.8.

Figure 5.10.bcompares the [^{3}H]-nicotine binding to beads after exposure to P2b preparations. The specific binding is given in the raw data form of cpm/mg protein in the tissue preparation. Binding to beads (2) and (4) are not significantly different at the 0.05 level (t-test, t=0.25, P=0.809)

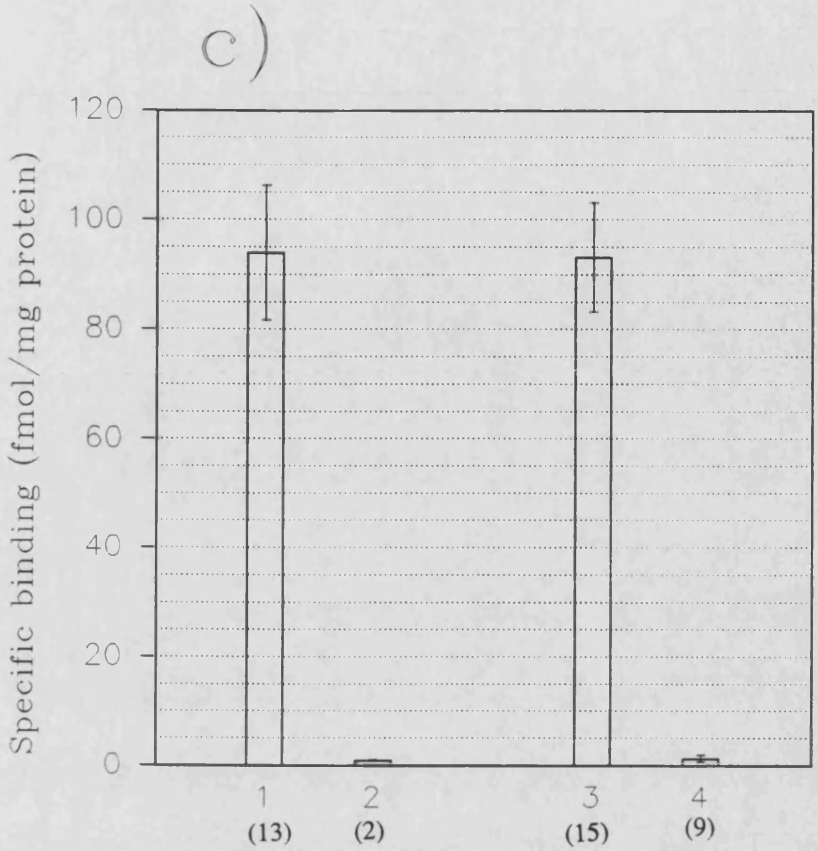


Figure 5.10.cdisplays the specific binding to both preparations and beads in fmol/mg of protein in the relevant P2b preparation.

Binding to preparations (1) and (3) are not significantly different at the 0.05 level (t-test, t=0.0536, P=0.958).

Binding to beads (2) and (4) are not significantly different at the 0.05 level (t-test, t=0.25,P=0.809)

the value of:-

$$\frac{\text{P2b preparation exposed to mAb coated beads} \times 100\%}{\text{P2b preparation exposed to control beads}}$$

provides a useful graphical method to compare the two preparations. Statistical analysis (t-test) indicates that there was no difference between exposed and control P2b preparations (see figure 5.10c).

5.3.4 Immunoisolation of material containing nAChRs from P2b preparations containing Triton

Immunoisolation experiments which were performed in the presence of Triton could not be measured using LDH measurement, because the detergent would release $LDH_{occluded}$, and also [^{3}H]-nicotine binding was a more sensitive approach for the detection of nAChR immunoadsorption.

5.3.4.1 Optimization of conditions

The effect of Triton concentration upon specific [^{3}H]-nicotine binding was indicated by the results of binding to dilution series of detergent extracts (figure 5.6, section 5.3.2.1). The effect of Triton concentration could be more readily investigated by measuring specific binding to nAChRs in the presence of Triton (figure 5.11). At concentrations of greater than 0.5% v/v Triton the solution viscosity was so great that its passage through the filter was retarded. The curve shown in figure 5.11.b provided a guide to projecting true specific [^{3}H]-nicotine

Figure 5.11 The effect of Triton concentration upon the level of detectable specific binding to P2b preparation

a)

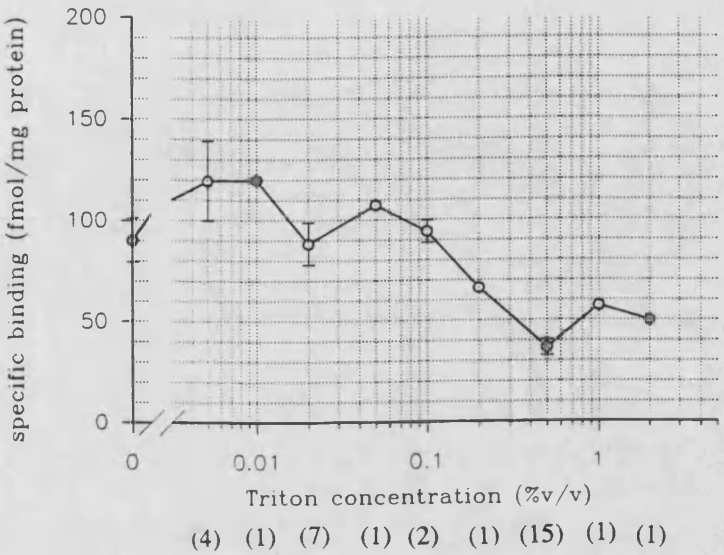


Figure 5.11.adisplays the results of [^{3}H]-nicotine binding (specific binding in fmol/mg protein) to P2b preparations in the presence of a range of Triton concentrations. SEMs shown, n displayed in brackets. Note, binding to P2b, 0% Triton, n = 15. Mean = 90.3 fmol/mg protein (SEM = 10.8)

b)

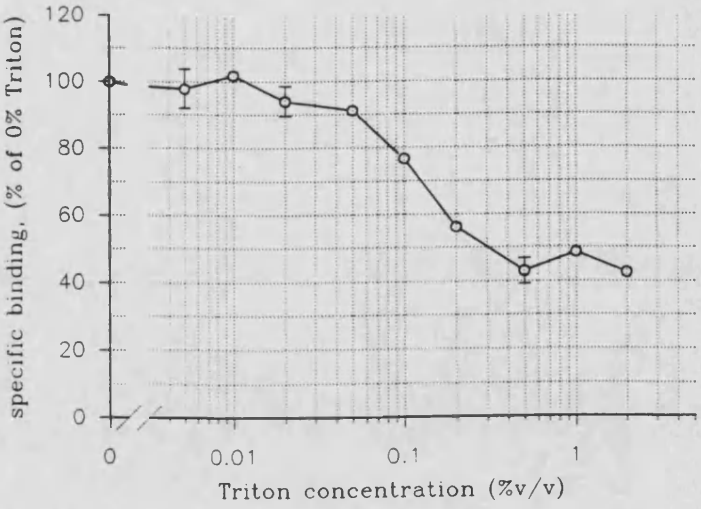


Figure 5.11.bdisplays the results of [^{3}H]-nicotine binding to P2b preparations in the presence of a range of Triton concentrations expressed in each experiment as a percentage of the specific binding to the experimental control, ie the P2b preparation containing 0% Triton. SEMs shown. n's as 4.11.a

binding from the apparent binding in the presence of Triton, however where possible paired controls were used in immunoisolation experiments, or, if necessary, all filtration measurements in an experiment were performed at a constant Triton concentration (eg 0.25% v/v): these 2 approaches avoid problems due to inaccurate measurement of Triton or pump variation.

5.3.4.2 Immunoisolation experiments

The effect of Triton concentration upon immunoadsorption using the Lindstrom mAbs is shown in figure 5.12. In this figure the trend which was indicated in figure 5.8 (section 5.3.2.2) was reinforced, ie that immunoadsorption increases with increasing Triton concentration, at least up to 0.5% v/v Triton. The experiment displayed in figure 5.8 was repeated using P2b preparation containing Triton instead of detergent extracts, the immunoadsorption of nAChR (figure 5.13) was very similar. The adsorption of nAChRs from detergent extracts (figure 5.8) in the presence of 0.5% Triton was 1.85 times the immunoadsorption from detergent extracts in the presence of 0.05% Triton, immunoadsorption from P2b preparations in the presence of Triton (figure 5.13) at 0.5% was 2.25 times the level of immunoadsorption with 0.05% Triton.

Experiments were performed to examine the effect of collagenase upon immunoadsorption of nAChR bearing particles from P2b preparations in the presence of 0.5% v/v Triton (figure 5.14). These experiments acted as controls for parallel experiments in the absence of Triton (section 5.3.5). Collagenase was found to lower specific binding slightly, but once this was compensated for, no other effect of collagenase was apparent.

Figure 5.12 **The effect of Triton concentration upon nAChR adsorption**

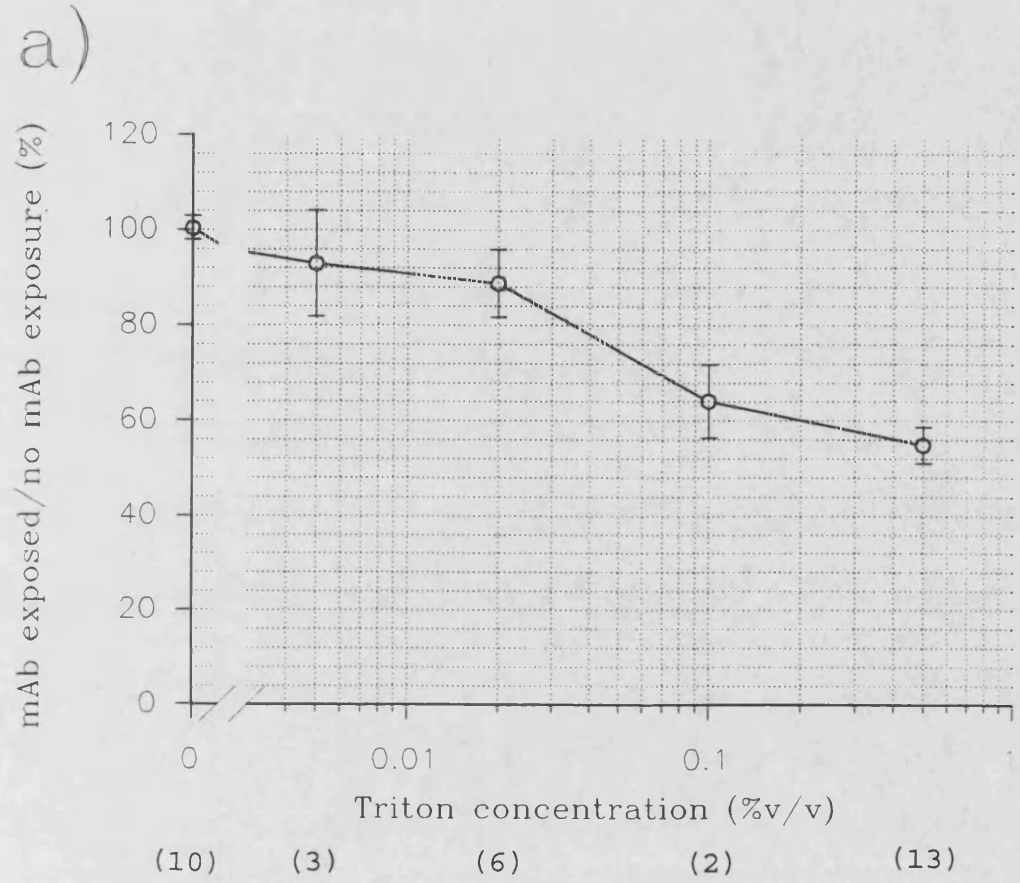


Figure 5.12.adisplays the ratio of [^{3}H]-nicotine binding to P2b preparations which had been exposed to mAb coated beads (50μl of 3%w/v) to the binding to P2b preparations which had been exposed to control beads alone, in the presence of a range of Triton concentrations, ie specific binding to

P2b preparations exposed to mAb coated Sepharose x 100%

P2b preparations exposed to control beads

SEMs shown, n displayed in brackets.

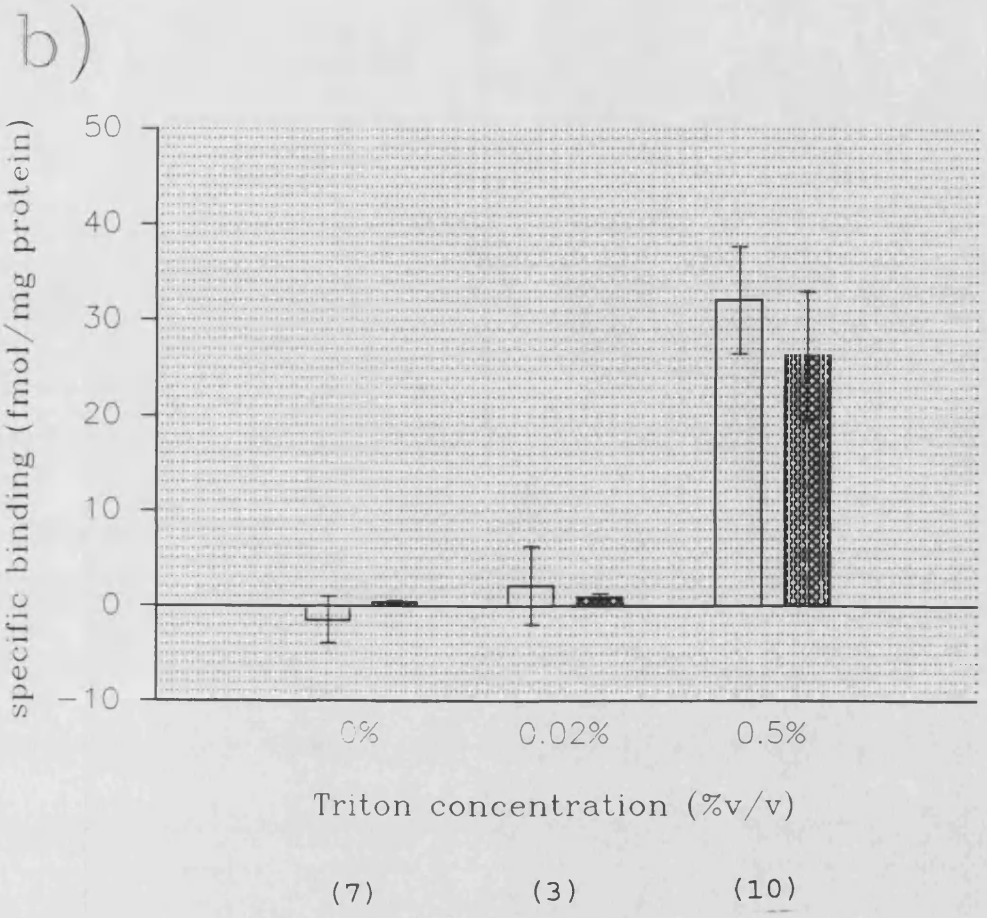


Figure 5.12.b displays the loss of specific binding (fmol/mg protein, ▭) from P2b preparations which have been exposed to Sepharose--anti-(rat IgG)--mAb relative to P2b preparations which have been exposed to Sepharose--anti-(rat IgG). The figure also displays the gain of specific binding (fmol/mg protein in relevant P2b preparation, ▩) by Sepharose--anti-(rat IgG)--mAb during exposure to the P2b preparation relative to Sepharose--anti-(rat IgG) controls during exposure to an identical P2b preparation. The transfer of nAChR from P2b preparation to mAb coated beads is shown at differing Triton concentrations. SEMs shown. n (brackets) is the same for loss from P2b preparations and gain by beads at any specific Triton concentration.

Figure 5.13 **The detection of the transfer of nAChRs from a P2b preparation to mAb coated Dynabeads, the effect of Triton concentration**

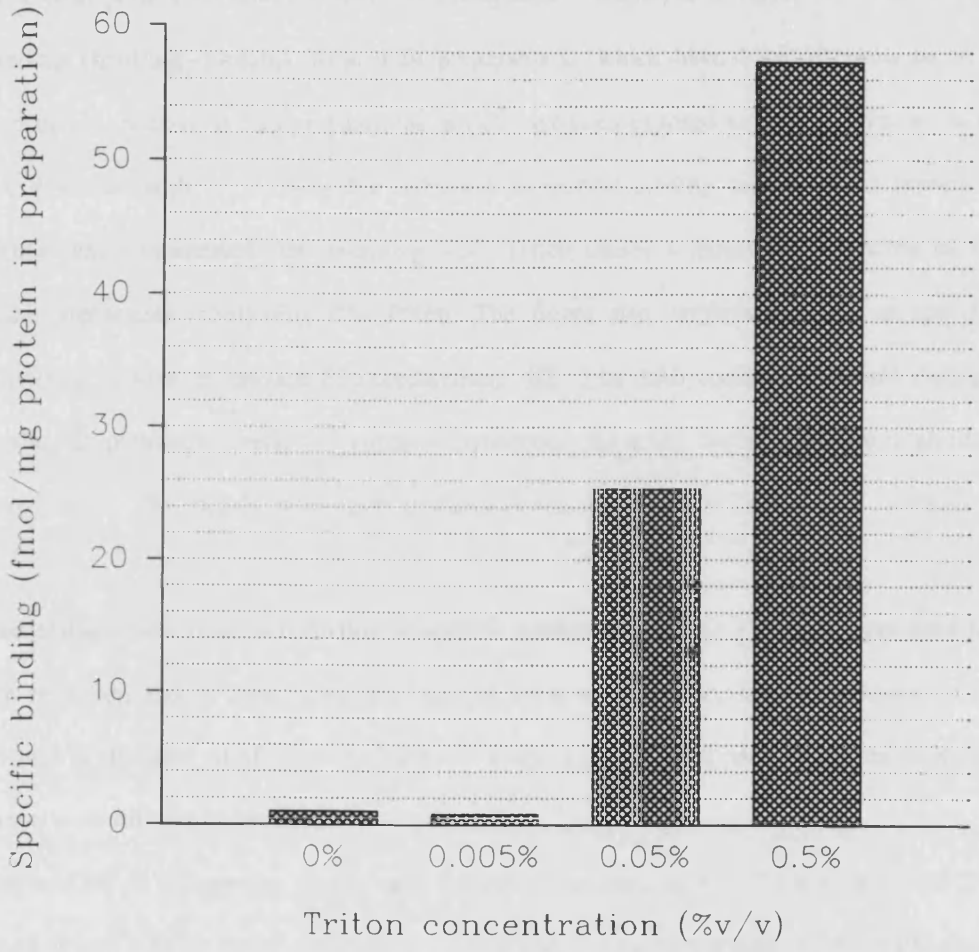


Figure 5.13 displays the results of a single experiment to compare with the results shown in figure 5.8.n=1. The experimental procedure used was identical to that described for figure 5.8, with the exception that the nAChR was depleted from a P2b preparation, in the presence of a range of Triton concentrations and not a detergent extract of P2 membranes. The specific binding to the mAb coated Dynabeads (50μl of 3%w/v) is shown in fmol [^{3}H]-nicotine binding/mg protein in the relevant P2b preparation.

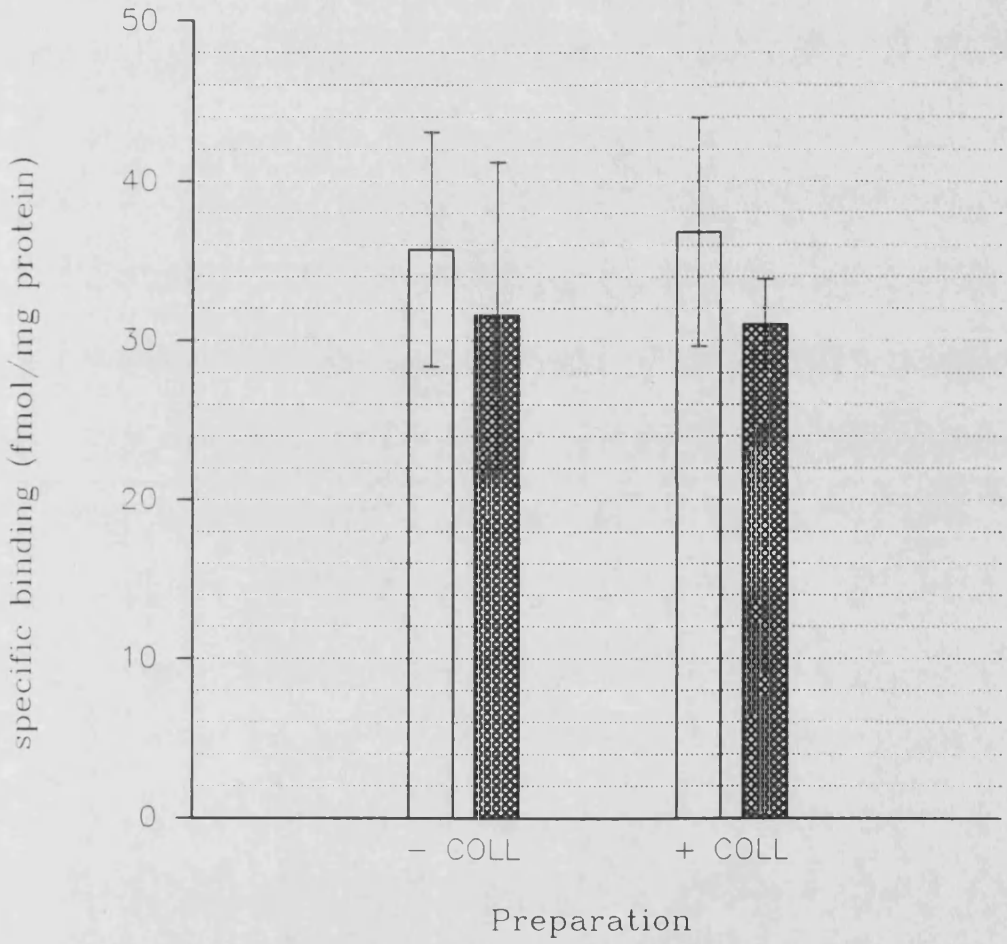
Figure 5.14 The effect of collagenase treatment upon the immunoadsorption of nAChRs from P2b membranes in the presence of 0.5% Triton

The transfer of nAChR to mAb coated beads from P2b preparations containing 0.5% Triton which have been pretreated with 0% or 0.1% collagenase is displayed in figure 5.14. The loss of specific binding (fmol/mg protein) from P2b preparations, which have been exposed to mAb coated Dynabeads, relative to P2b preparations which have been exposed to control Dynabeads (no mAb), are displayed with ☐. Note the reduction in specific binding caused by the presence of 0.5% Triton was compensated for, assuming 0.5% Triton causes a reduction of binding to 43% of the same preparation containing 0% Triton. The figure also displays the gain of specific binding (fmol/mg protein in relevant P2b preparation, ▨) by mAb coated Dynabeads during exposure to the P2b preparation relative to control Dynabeads (no mAb) during exposure to an identical P2b preparation. The specific binding to Dynabeads was measured in Tris/HEPES without Triton.

The collagenase caused a reduction in specific binding (to 84.5%). Controls were used to measure the reduction and to compensate for that reduction separately for each experiment (n = 4 in all cases, SEM shown). Statistical analysis (t-test of all pairs) indicates (not shown) that there is no difference between:

lost nAChR in collagenase treated and control preparation at the 0.05 level ($t=0.107, P=0.918$).

Bead bound nAChR from collagenase treated and control preparation at the 0.05 level ($t=0.107$, $P=0.918$).


50
40
30
20
10
0
specific binding (fmol/mg protein)
– COLL
+ COLL
Preparation
Key
specific binding lost during bead exposure
specific binding to beads after exposure

5.3.5 Attempts to achieve immunoisolation of subcellular particles bearing nAChRs from synaptosome preparations in the absence of Triton

Triton could not be used to immunoisolate subcellular particles (eg synaptosomes) bearing nAChRs, because it disrupts membranes. Instead, 4 approaches to exploring probe access or probe dissociation were examined (see section 5.1 for rational):-

a) The P2b preparation was pretreated with collagenase, sometimes in the absence of protease inhibitors, before immunoadsorption.

b) The P2b preparation was pretreated with glycosidase before immunoadsorption.

c) The P2b preparation was exposed to biotinylated mAbs and then the mAb exposed synaptosomes were incubated with Avidin coated Dynabeads. Washing was performed by repeated addition of buffer in the absence of a magnetic field and buffer removal after bead collection in the magnetic field.

d) As (c) but cross-linking mAbs to nAChR by fixation using 1% glutaraldehyde fixation (room temperature, 1h). Immunoadsorption proceeded with very gentle agitation - every 15min the vial was turned end-over-end once. Washing was also very gentle washing, beads were held in a constant magnetic field and buffer added then removed, so that the passage of buffer over the surface of the beads washed material away. Note some of the P2b preparation was washed away using this method, washing proceeded for 20 minutes, during which time approximately 2 thirds of the preparation was removed in all cases.

In all cases immunoisolation attempts were monitored by the more sensitive [^{3}H]-nicotine binding assays.

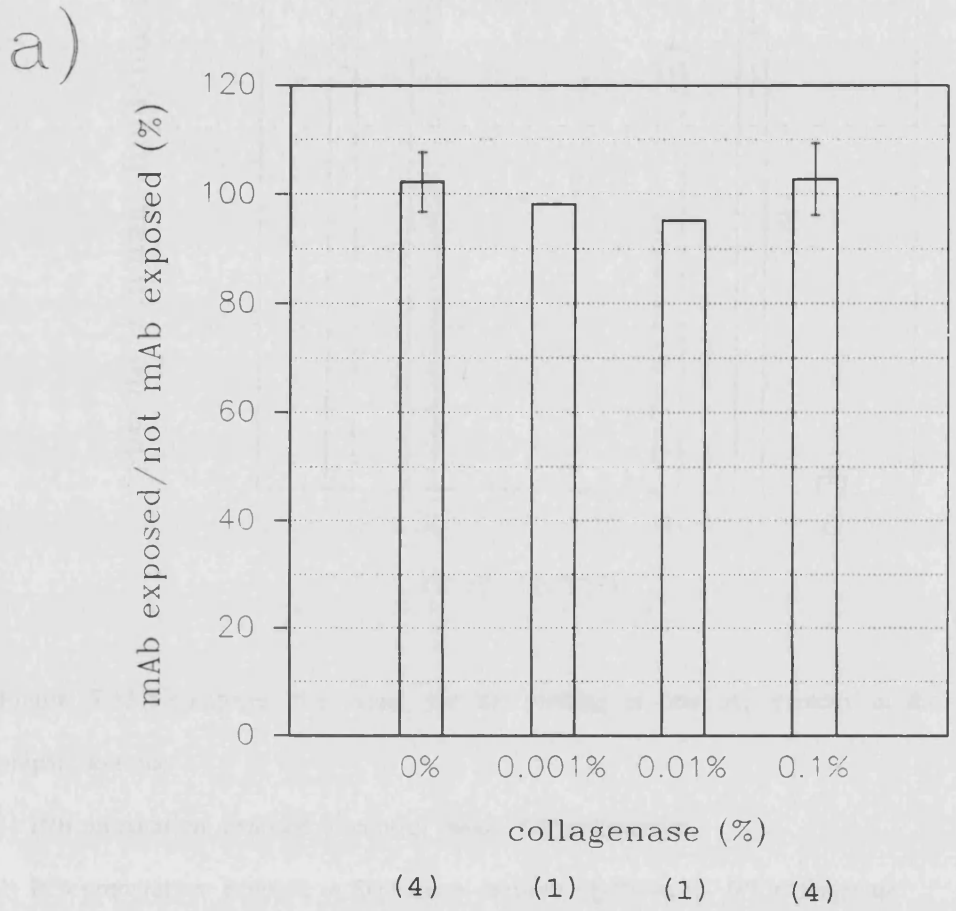
a) Collagenase treatment caused a slight reduction in specific binding to preparation, but no immunoadsorption of subcellular particles (figure 5.15): there was no depletion by mAb coated beads of nAChRs from P2b preparations after treatment by a range of collagenase concentrations and no gain of nAChRs by beads.

b) There was no depletion by mAb coated beads of nAChRs from P2b preparations after pre-treatment with glycosidase and no gain of nAChRs by beads (figure 5.16). Glycosidase treatment seemed to have no effect upon ligand binding to P2b preparations.

c) Immunoisolation of subcellular particles bearing nAChRs was not improved by attempting to coat the synaptosomes with mAbs and then exposing those synaptosomes to beads (figure 5.16)

d) When glutaraldehyde fixation experiments were performed material bearing nAChRs was present with the Dynabeads (figure 5.16), however the precipitation was non-specific and not due to immunoadsorption.

Figure 5.15 **The effect of collagenase treatment upon the immunoadsorption of nAChRs under physiological conditions**



P2b preparations were pre-treated with a range of collagenase concentrations in PBS, for 1h, at room temperature. Figure 5.15.a compares [^{3}H]-nicotine binding of P2b preparations were then exposed to mAb coated Sepharose, with P2b preparation exposed to controls (no mAbs), ie:-

$$\frac{\text{P2b exposed to Sepharose--anti-(rat IgG)--mAb}}{\text{P2b exposed to Sepharose--anti-(rat IgG)}} \times 100\%$$

Statistical analysis (t-test) indicates there is no difference between 0% and 0.1% collagenase treatment at the 0.05 level ($t=0.0588, P=0.956$). SEMs shown, n in brackets.

b)

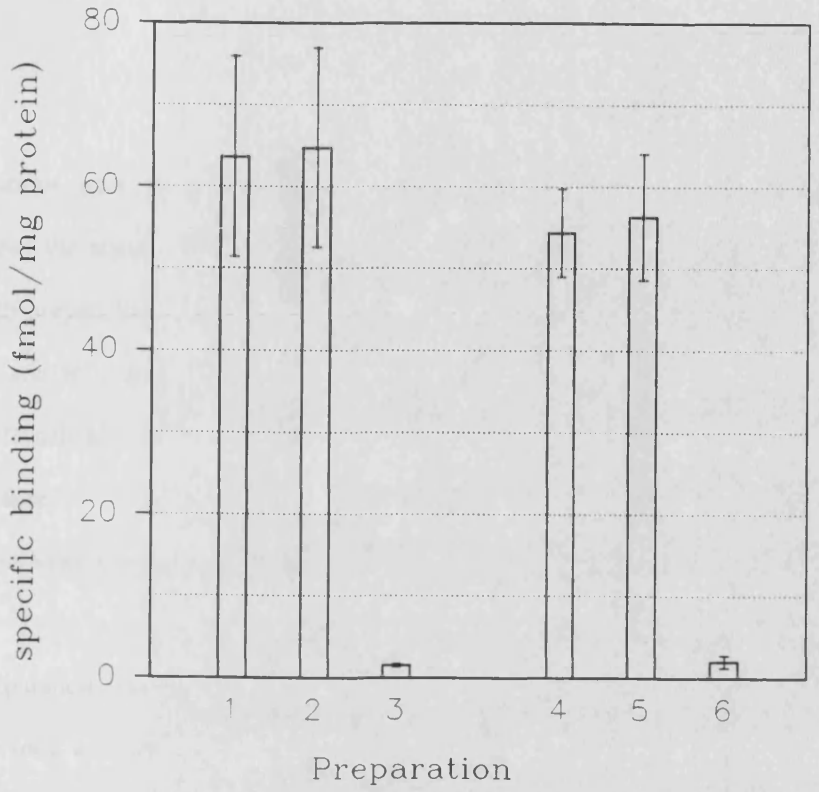


Figure 5.15.b compares the actual specific binding in fmol/mg protein in the relevant P2b preparation to:-

1) P2b preparation exposed to control beads, 0% collagenase

2) P2b preparation exposed to Sepharose--anti-(rat IgG)--mAb, 0% collagenase

3) Sepharose--anti-(rat IgG)--mAb after exposure to P2b preparation (2), 0% collagenase

4) P2b preparation exposed to control beads, 0.1% collagenase

5) P2b preparation exposed to Sepharose--anti-(rat IgG)--mAb, 0.1% collagenase

6) Sepharose--anti-(rat IgG)--mAb after exposure to P2b preparation (5), 0.1% collagenase

SEMs shown. n=4 throughout. Statistical analysis (t-test) indicates there is no difference between:-

(1) and (2) at the 0.05 level ($t=0.0636, P=0.951$)

(4) and (5) at the 0.05 level ($t=0.207, P=0.843$)

(3) and (6) at the 0.05 level ($t=0.584, P=0.580$)

Figure 5.16 The effect of alternative approaches to the immunoadsorption of nAChRs from P2b preparations in the absence of Triton

P2b preparations were pre-treated with or without glycopeptidase F in PBS, for 4h, at 37°C. Figure 5.16 compares the actual specific binding (SEMs shown, n's in brackets) in fmol/mg protein of the relevant P2b preparation. Beads were used at 50μl of 3%w/v.

1) P2b preparation exposed to control beads (2), after pretreatment with 0% glycosidase

2) Control Dynabeads (no mAb) after exposure to the P2b preparation (1), after pretreatment with 0% glycosidase

3) P2b preparation exposed to control beads (2), after pretreatment with 0.5% v/v glycosidase (one unit/ml)

4) P2b preparation exposed to mAb coated Dynabeads (5), after pretreatment with 0.5% v/v glycosidase (one unit/ml)

5) Dynabeads coated with mAbs after exposure to P2b preparation (4), after pretreatment with 0.5% v/v glycosidase (one unit/ml)

6) Dynabead-Avidin after incubation with P2b preparation which had been pre-exposed to biotinylated mAbs

7) Dynabead-Avidin after incubation with P2b preparation which had been pre-exposed to biotinylated mAbs, then fixed with 1% v/v Glutaraldehyde. NB minimal agitation during incubation and minimal washing

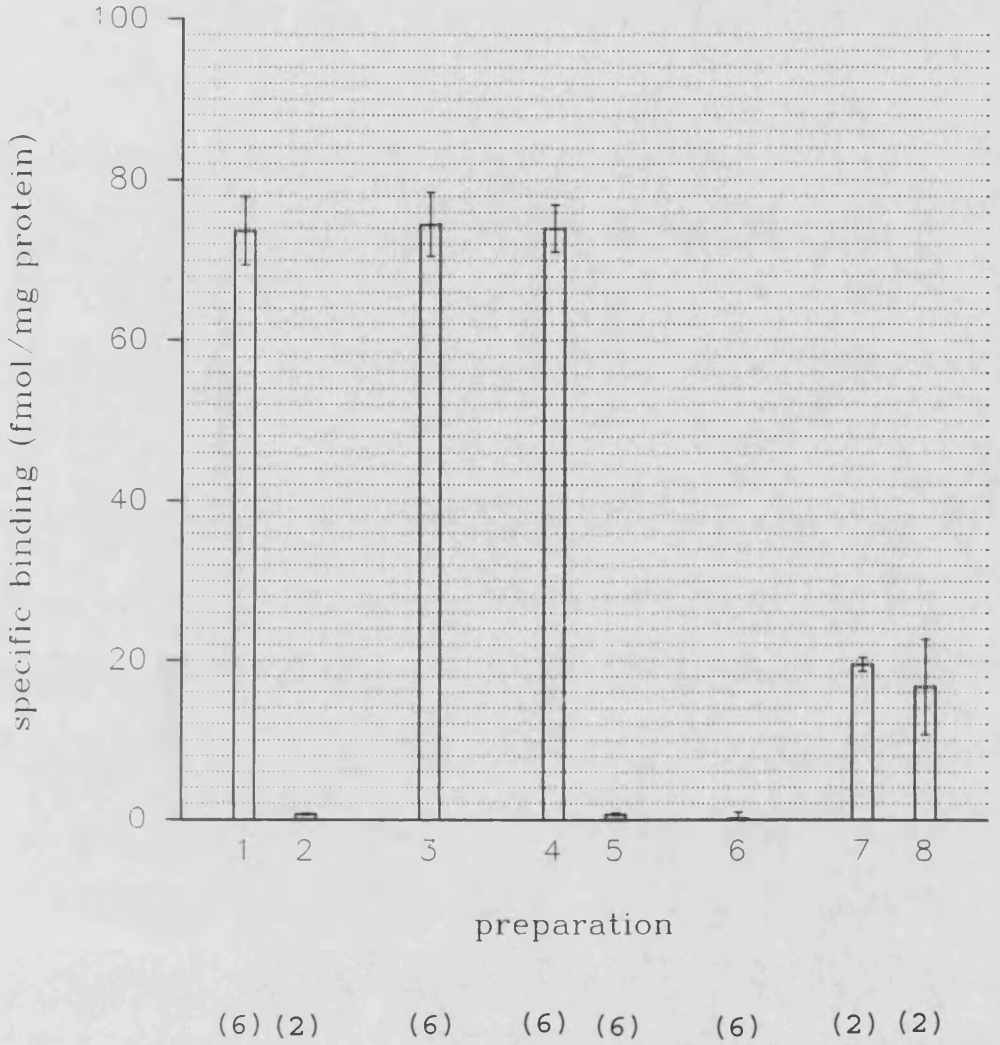
8) Dynabead-Avidin after incubation with P2b preparation which had been not been pre-exposed to biotinylated mAbs, but then had been fixed with 1% v/v Glutaraldehyde. NB minimal agitation during incubation and minimal washing

Statistical analysis (t-test of all pairs) indicates (not shown) that there is no difference between:

(1), (3) and (4) at the 0.05 level

(2), (5) and (6) at the 0.05 level

(7) and (8) at the 0.05 level


specific binding (fmol/mg protein)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
preparation
(6) (2) (6) (6) (6) (6) (2) (2)

5.4 Discussion

5.4.1 Adsorption of antibodies to beads

The failure to detect bead bound mAbs with a phosphatase linked probe was not fully investigated, once the peroxidase linked system was established. The results, described in section 5.3.1,only indicate recognition failure using a single batch of phosphatase linked probe. Different batches of phosphatase linked probe would probably be suitable for mAb detection, because the same methods are used to raise antisera for both types of enzyme linked probe (Sigma technical information). The possible explanations for recognition failure for this batch are numerous, they range from accidental probe denaturation to a gap in the repertoire of epitopes presented to the immunological response.

The direct and indirect attachment of mAbs to beads could be detected, using several batches of peroxidase probe. The relative merits of direct and indirect attachment for the immunoadsorption of subcellular particles were explored by Richardson and Luzio (1986, 1988). Direct attachment has 2 advantages: secure attachment and specificity because there are no other species of probe present. Indirect attachment is favoured, it has the following advantages:-

i) the orientation of presentation is optimal, this dramatically increases the number of available recognition sites,

ii) steric problems are reduced by using a longer linkage between the beads and subcellular particles,

iii) conformational changes in the primary probe due to attachment are less likely.

The verification of the direct attachment of all 4 mAbs confirms that each mAb could be recognized when bound to the matrix using the peroxidase probe. Indirect attachment of mAbs via anti-rat IgG linkers and of biotinylated mAbs via Avidin linkers was verified confirming successful mAb recognition by the linker. Protein A (2 batches) and Protein G (1 batch) were used as linker proteins for indirect attachment, but neither could be used to adsorb any of the mAbs directly. The two linker proteins were functional, eg immunoadsorption was successfully performed using a triple sandwich consisting of Sepharose--Protein G--anti-(rat IgG)--mAb. The recognition of rat IgGs by both Protein A and Protein G is often poor or non-existent (Sigma and Pharmacia, technical information) and so recognition failure was not surprising. In the case of Protein G, the problem may have again been limited to the batch used.

Two types of bead were used in immunoadsorption experiments:-

a) Sepharose 6MB beads were used for preliminary experiments, they were easily handled and could readily be separated by gravity. The diameter of beads (Sigma, technical information) was approximately 1000 times the diameter of synaptosomes (0.5 μm: Jones, 1975), when successful immunoadsorption occurs synaptosomes would be expected to coat the beads.

b) Dynabeads are 5-9 times the diameter of synaptosomes (M450 are 4.5 μm and M280 are 2.8 μm diameter: Dynal, technical information) to synaptosomes. They were originally used in the hope that the reduced size would enable the formation of a matrix of Dynabeads and synaptosomes, where immunoadsorption might have been more efficient, because the number of sterically available attachment sites for synaptosomes bearing nAChRs would be far higher, and so the probability of

detachment would be far lower (Richardson and Luzio 1986, 1988). However this hypothesis was contradicted by empirical observations (Docherty *et al*, 1991) which indicate that Dynabeads are a less efficient immunoadsorbant of synaptosomes than larger magnetic beads (Magnogel, 60-140μm diameter). Dynabeads can be collected very gently by magnetic separation or more vigorously by centrifugation (Docherty person. comm.; Dynal, technical information), the advantage of gentle separation was that fewer attachment bonds would be broken. Magnetic collection can suffer from washing problems, because the beads are not dense and could easily be washed away (Brennand D., person. comm.), this meant that careful washing by magnetic collection was labour intensive, and controls were always performed. The attachment of biotinylated mAbs to Avidin-coated beads had potential advantages because of the specificity and affinity of the Avidin-biotin bond (appendix A.3).

5.4.2 Immunoadsorption of nAChRs from detergent extracts of P2 membranes

The mAbs were raised and selected by their ability to recognize nAChR in 0.5% v/v Triton extracts (Fraser and Lindstrom, 1984; Lindstrom, 1984, 1986; Lindstrom *et al*, 1981, 1987), thus in this investigation the activity of attached mAbs was confirmed by showing successful immunolocalization of nAChRs from detergent extracts (Whiting and Lindstrom 1986a, 1987). The detection of immunolocalization was aided by the fact that high affinity [^{3}H]-nicotine binding sites detected in the filtration assay (section 3.2.6)correspond to the α4ß2 subtype (section 1.3). The recovery of protein during detergent extraction was approximately 28%, this is comparable to previous studies (30-50%: Newby, 1984).

5.4.2.1 Characterization of [^{3}H]-nicotine binding assay

In the dilution experiments displayed in figure 5.6 both Triton and protein were diluted because there was no other method of rapidly and predictably altering the Triton concentration (Lindstrom, 1985): this means that the error in protein estimation increased, and the measured level of specific binding decreased, with decreasing Triton concentration. These two factors explain why the variance in specific binding (fmol/mg protein) was expected to increase with decreasing Triton concentration. The variance was too great realistically to apply statistical analysis to verify the trend seen in figure 5.6, ie that there was a decrease in detectable specific binding with increasing Triton concentration. However, the same trend was seen when Triton was added to P2 membranes (figure 5.11, section 5.3.4.1) and so there is no reason to believe that the trend implied in figure 5.6 does not occur.

Triton X-100 reversibly binds to *Torpedo* nAChRs and (Suárez-Isla and Hucho, 1977) non-competitively inhibits [^{3}H]-nicotine binding (Heidmann *et al*, 1983). Triton X-100 does not seem to inhibit [^{3}H]-nicotine binding to the $\alpha 4\beta 2$ receptor, eg dialysis (Abood *et al*, 1980) and immunoprecipitation assays show no loss of [^{3}H]-nicotine binding to Triton solubilized receptors (sections 3.3.3, 3.3.4 and 3.4.3;Whiting and Lindstrom 1986a). The deterioration in nAChR detection with increasing Triton concentration observed in sections 5.3.2 and 5.3.4.1 probably reflects an increase in the number of nAChRs which pass through the filter with increasing solubilization. This phenomenon is observed using the filtration assay for the measurement of the binding of ligands to many solubilized receptors and the loss for the [^{3}H]-nicotine binding assay is relatively low (Bruns *et al*, 1983).

Care was taken throughout immunoadsorption experiments to measure [^{3}H]-nicotine binding at reproducible and minimal Triton concentrations (eg Whiting and Lindstrom, 1986a), the concentration of Triton was reduced by dilution because of the predictability of this approach (see the start of this section).

5.4.2.2 The results of immunoisolation experiments aimed at the adsorption of nAChRs from detergent extracts of P2 membranes

Each of the 4 mAbs were individually used to immunoadsorb nAChRs from a detergent extract of P2 membranes (section 5.3.2.2),essentially as described by Whiting and Lindstrom (1986a, 1987). The level of immunoadsorption was increased in one set of parallel experiments (Treatments 5 and 6), by increasing the temperature and decreasing the degree of agitation. After demonstrating that each mAb could immunoadsorb nAChRs separately, all further experiments were performed using a mAb cocktail to maximize recognition and affinity (Richardson and Luzio 1986, 1988).

Immunoadsorption could not be measured by the method described in section 5.3.2.2.b,ie acid dissociation followed by the filtration assay. Acid dissociation may or may not cause receptor dissociation, but it does seem to alter receptor conformation such that specific [^{3}H]-nicotine binding is no longer possible, as implied by Lunt (1987).

Immunoadsorption could be monitored directly as an increase in specific [^{3}H]-

nicotine binding associated with beads (Whiting and Lindstrom, 1986a), or indirectly by measuring the loss of specific [^{3}H]-nicotine binding from extracts (Whiting and Lindstrom, 1986a). The importance of Triton for nAChR recognition was demonstrated by the observation that immunoadsorption increased with increasing Triton concentration. Very little of the nAChR was lost during transfer from detergent extracts onto the beads. The small quantities of nAChR which were not recovered probably reflect the dissociation of nAChRs from the beads during washing. This deduction was reinforced by the observation that there was more missing material from the beads which had been exposed to 0.05% Triton than to 0.5% Triton, suggesting that the strength of attachment was reduced at the lower Triton concentration.

5.4.3 The immunoadsorption of nAChR bearing material from P2b preparations

Measurement of LDH activity in homogenate indicates some cytoplasm is released (36%) during homogenization, but considerable cytoplasm remained occluded in resealed vesicles. The concentration of non-occluded LDH in Hb preparations was negligible, reflecting the fact that little lysis of subcellular particles had occurred after all free LDH had been washed away; under appropriate conditions synaptosomes tend to be fairly stable (Bradford *et al*, 1975). The controls indicated that no other dehydrogenases were measured in these experiments. The occluded LDH was gradually released with storage reflecting the disruption of subcellular particles. The low level of LDH activity associated with beads seems entirely due to inadequate washing, indicating that no immunoadsorption had occurred. It was possible that immunoadsorption had

occurred at a level which was too low for detection, but the more sensitive [^{3}H]-nicotine binding assay was capable of detecting very low levels of immunoisolation. The failure to detect immunoadsorption of subcellular particles bearing nAChRs from P2b preparations using [^{3}H]-nicotine binding assays can be interpreted in a number of ways, which were addressed by the immunoisolation experiments described in sections 5.3.4and 5.3.5:-

Interpretation a) Immunoadsorption did occur, but remained undetected, for example this might reflect inhibition of [^{3}H]-nicotine binding by the binding of mAbs, or immunoadsorption at a very low level. Neither possibility is likely. Immunoadsorption would have to be at a very low level not to be detected by the [^{3}H]-nicotine binding assays. There is no indication in any Lindstrom literature that the 4 mAbs block [^{3}H]-nicotine binding (Lindstrom *et al*, 1987), there was no depletion of nAChR from the P2b preparations due to mAb exposure (section 5.3.3.b)and immunoadsorption of detergent solubilized receptors does not block binding (sections 5.3.2.2and 5.4.2.2).

Interpretation b) Immunoadsorption did not occur, because the nAChRs were blocked by other proteins, which were altered or removed during Triton exposure to allow antibody recognition. Blocking might be caused by the synaptic junctional matrix (Cotman and Taylor, 1972) or by the extracellular matrix (Froehner, 1993; Reichardt and Tomaselli, 1991). Blocking by the synaptic junctional matrix was evaluated by measuring [^{3}H]-nicotine binding to the SJC (section 3.4.2), it does not account for the failure of antibodies to recognize all nAChRs. The P2b preparations were pre-treated with collagenase in the absence of protease

inhibitors before immunoadsorption; this addresses the possibility of protection in 2 ways:-

i) The collective pool of proteases, including collagenase, might digest extracellular blocking proteins (Reichardt and Tomaselli, 1991) and allow immunoadsorption. No immunoadsorption was observed indicating either inadequate digestion or evidence against interpretation (b). Collagenase also had no effect upon immunoadsorption from P2b membranes in the presence of 0.5% v/v Triton (section 4.4.6.2), this observation contradicts the possibility that immunoadsorption failed because of inadequate digestion: if Triton and proteases both acted partially to remove masking proteins their effects would be additive and Triton would have more easily revealed nAChRs after the protease digestion, increasing immunoadsorption.

ii) Proteins which block antibody binding might be expected to prevent, or reduce, the protease digestion of the nAChR. The use of these proteases did result in the partial digestion of nAChRs, indicating at least partial access of enzymes to nAChRs. Furthermore, digestion did not increase when 0.5% v/v Triton was added to the P2b membranes (section 4.4.6.2), which would be expected to remove the antibody blocking protein according to this interpretation. If antibody blocking proteins were present they did not block protease activity.

Far stronger evidence against interpretation (b) has already been evaluated in section 5.3.2.2(see interpretation f). The experiment to show immunoadsorption of nAChRs from detergent extracts to Dynabeads at different Triton concentration was performed using a single detergent extract, which was diluted ten fold with either Tris/HEPES buffer, or Tris/HEPES buffer containing 0.5%

Triton. If interpretation (b) had been correct nAChR access would have been identical in both preparations, because the degree of blocking protein removal was the same.

Interpretation c) Immunoadsorption did not occur, because the method used tended to be too disruptive to linkage between beads and subcellular particles (Richardson and Luzio 1986; 1988): immunoadsorption did occur in the presence of Triton because the micelles which were produced were too light to cause dissociation. This is a difficult interpretation to disprove, even successful immunoadsorption experiments can have low yields (Richardson *et al*, 1984; Richardson and Luzio 1986)! Immunoadsorption was altered as follows:-

i) The temperature of immunoadsorption was increased to room temperature (2-4h).

ii) The P2b preparation was incubated with biotinylated mAbs (room temperature, 2-4h) before exposure to Avidin coated Dynabeads. This method of indirect attachment was recommended for the immunoisolation of subcellular particles (Richardson and Luzio 1986; 1988), because there is a greater probability of bead attachment to nAChR attached mAbs than to the nAChRs themselves. The high affinity of Avidin-biotin binding decreased the probability of loss of attachment.

iii) Immunoadsorption was favoured by gentle end-over-end agitation and magnetic collection (Docherty *et al*, 1987a; Richardson and Luzio 1986, 1988).

iv) The weakest linkage was the mAb to nAChR bond, this attachment was reinforced by glutaraldehyde fixation, before adsorption to Avidin coated beads.

None of these changes allowed specific immunoprecipitation to occur (section 5.3.5).The high level of non-specific precipitation in the final experiments showed that conditions could not be made more gentle! Stronger evidence that dissociation was not responsible for the failure of immunoadsorption was provided by the results of the immunostaining experiments (section 3.4.7and 4.4).

Interpretation d) The nAChRs are internal (Hill *et al*, 1993; section 4.4.1.1,4.4.2) and are released by synaptosomal lysis due to Triton exposure (Docherty *et al*, 1987a). This interpretation can be rejected for 2 reasons:-

i) The level of immunoadsorption differs from detergent solubilized material of 2 different Triton concentrations, which were made from a common detergent extract. Any release of internal nAChRs would have been the same.

ii) Immunoadsorption from extracts at different Triton concentrations mirrors immunoadsorption from P2b preparations in the presence of those Triton concentrations, this does not disprove interpretation (d), but it does tend to contradict it.

Interpretation e) The nAChR are dispersed and very few receptors are present on each synaptosome, so that immunoadsorption does not occur, because the few attachments which are made are insufficient to prevent disruption. It is known that immunoadsorption is improved by increases in points of attachment (Richardson and Luzio 1986; 1988), however this cannot be the explanation for these results because immunostaining also failed (section 3.4.7).

Interpretation f) Triton causes a change in the protein conformation of the

nAChR or possibly the mAbs, which allows the recognition of the nAChRs by the mAbs. In the 1970's, Triton X-100 was shown to bind to *Torpedo* nAChRs and induce conformational changes, which are not caused by other detergents (Suárez-Isla and Hucho, 1977): the Triton X-100 acts as a noncompetitive inhibitor of high affinity ligand binding (Heidmann *et al*, 1983). Perhaps the Triton X-100 has a similar effect upon $\alpha 4\beta 2$ receptor, certainly solubilization often causes some conformational changes (Lindstrom, 1985). The experiments described in section 5.3.4demonstrated the effect of Triton concentration upon immunoadsorption of nAChRs from P2b preparations. Immunoadsorption increased with increasing Triton concentration, as determined by depletion of the specific [^{3}H]-nicotine binding from P2b preparations, and by the gain of specific [^{3}H]-nicotine binding by beads (section 5.3.4.2). The experiments, which were performed to show immunoadsorption of nAChR from detergent extracts using Dynabeads (section 5.3.2.2),were repeated using P2b preparations containing Triton (section 5.3.4.2). The effect of Triton concentration upon immunoadsorption was the same in both experiments.

The evidence presented in this thesis strongly supports interpretation (f). Two methods were employed to try to induce conformational changes allowing receptor immunoadsorption in the absence of Triton (section 5.3.5):-

i) Protease exposure. Protease exposure can cause conformational changes resulting in loss of [^{3}H]-nicotine binding (section 5.3.5, 5.3.4.2; Whiting and Lindstrom, 1986b, c), but conformational changes which aided immunoadsorption were not detectable. If digested receptors were attached to beads their concentration was low, because the level of undetected nAChR was low (section 5.3.5,5.3.4.2).

ii) Glycosidase exposure. Experiments were performed to eliminate the possibility that the binding of mAbs to nAChRs in the absence of Triton was blocked by the nearby glycosylation sites (Anand *et al*, 1991; Gehle and Sumikawa, 1991; Nomoto *et al*, 1986). This hypothesis was unlikely, but it was investigated for completion. Exposure to glycopeptidase F was known to remove the carbohydrate component of the $\alpha 4\beta 2$ receptor (Anand *et al*, 1991). Attempted deglycosylation had no effect upon immunoadsorption so this approach was not pursued.

5.4.4 The viability of immunoadsorption of synaptosomes bearing nAChRs

The experiments interpreted in section 5 demonstrate that immunoadsorption of synaptosomes bearing the nAChR was not possible using the 4 Lindstrom mAbs, this contradicts the preliminary results described by Irons *et al* (1988, 1989). The immunoadsorption results strongly indicate that receptor recognition by the monoclonal antibodies was dependent upon Triton concentration, the reason for this seems to be that Triton causes conformational changes in the nAChR or possibly the mAbs which are a prerequisite for recognition by the 4 mAbs. No alternative treatment was discovered which allowed nAChR immunoadsorption in the absence of Triton.

Immunoadsorption was measured by high affinity [^{3}H]-nicotine binding to bead bound nAChR and non-adsorbed nAChR. This technique is superior to measurement in preliminary experiments (Irons *et al*, 1988, 1989), which were based on the measurement of cytoplasmic markers and neurotransmitter synthesizing enzymes (Docherty *et al*, 1987b; Richardson *et al*, 1984). High affinity

[^{3}H]-nicotine binding is a sensitive technique, which measures $\alpha 4\beta 2$ (section 1.3) directly and so, in this case, it was a far more accurate approach for investigating immunoadsorption. All available evidence indicates that the immunoadsorption of synaptosomes bearing nAChRs is a viable objective, using other antibodies.

Chapter 6 Conclusions

6.1 Summary of results

Ligand binding to subcellular fractions (section 3) reinforced previous studies which demonstrated that α4ß2 nAChRs are present in synaptosomes (section 3.4.3).Preliminary ligand binding studies indicated that a large proportion of the α4ß2 isoform are not present in the SJC itself, where they might be protected by the synaptic junctional matrix (section 3.4.2).Thus, a large proportion of receptors would appear to be accessible for immunoadsorption (section 5.4.3).

Attempts to immunoadsorb synaptosomes bearing the nAChR were unsuccessful, but various experiments were performed to determine the reason for this (section 5.3.5).There did seem to be a large proportion of receptors which were accessible for immunological recognition (5.4.3). Immunostaining techniques were unsuccessful (section 4 and especially 3.4.7), thus the reason why immunoadsorption was not successful was not that the nAChRs were so widely dispersed that there were too few attachment sites on each synaptosome: if immunological recognition had occurred even widely dispersed nAChRs would have been detected by immunostaining (section 3.4.7).Sections 3.4.4,3.4.5,4 and 5.4.1 discuss how mAbs were shown to recognize the secondary probes; section 5.4.1 describes how mAbs were shown to attach to the bead matrix; section 5.4.2 describes how the bead-bound mAbs were successfully used to immunoadsorb nAChRs from detergent extracts of synaptosome preparations. However, immunological recognition of the α4ß2 isoform seemed to decrease with decreasing Triton concentration (sections 5.4.2.2 and 5.4.3). None of the treatments considered in section 5.4.3 altered immunological recognition; it is

possible that alternative treatments would be more successful. There are several possible explanations for the dependence of immunological recognition upon Triton: the explanation favoured by the author, based on the work presented in this thesis, is that Triton causes a conformational change in either the nAChR or the mAbs which enable immunological recognition (section 5.4.3). This explanation is proposed, because the mAbs were originally raised and screened against detergent solubilized nAChRs (section 1.4) and Triton X-100 has been shown to cause conformational changes in *Torpedo* nAChRs (section 5.4.3.f).No experiments have been performed to determine if this effect is limited to the rat $\alpha 4\beta 2$ isoform: it is possible that these mAbs do not require Triton for the recognition of other isoforms or even of the $\alpha 4\beta 2$ isoform of other species. No experiments were performed to determine if the dependence of immunological recognition upon Triton was caused by the storage conditions of the mAbs, which had been lyophilized for transport and were resuspended and frozen in aliquots for use within 6 months. The synaptosome immunolocalization experiments evaluated in section 5 are almost certainly viable, if antibodies which recognize the receptor in the absence of Triton X-100 can be found by the screening procedure derived from section 5.2 in appendix B. Monitoring immunoadsorption would be considerably aided by using antibodies which do not block high affinity [^{3}H]-nicotine binding. An alternative approach to correlating neurotransmitter content with nAChRs might be to perform [^{3}H]-nicotine binding to synaptosomes which have been shown to be immunopurified on the basis of expressed neurotransmitter-synthesizing enzymes. This possibility was not investigated because of time constraints.

Immunoelectron microscopy of synaptosomes is probably best performed by prefixation immunostaining (appendix A, section 4.4.1 and 4.4.3). The concentration of the α4ß2 nAChRs is so low (section 4.4.1) that detection in unpurified synaptosomes may not be possible; however, if this approach is viable, an extensive study is certainly required (section 4.4.3). Post-embedding immunoelectron microscopy is probably an inferior approach to the detection of the α4ß2 isoform in synaptosomes, unless attempts were made to detect cytoplasmic nAChR sites (section 4.4.2 and 4.4.3). Immunostaining controls (section 3.4.7) indicated that immunological recognition was not possible using the mAbs without Triton and so a primary aim of further immunoelectron microscopy of synaptosomes would have to be to find antibodies which would bind to the nAChR with high affinity under conditions which do not damage or prevent the detection of synaptosomal ultrastructure. Immunoelectron microscopy has been performed upon tissue blocks, and this is clearly an appropriate approach because identification is far easier, alternatively immunopurified synaptosomes could be analyzed as they would bear far higher nAChR concentrations. Both these approaches were not attempted because they compromised the original objectives of the project (section 4.4.3).

6.2 The role of presynaptic AChRs

This study reinforces previous studies which indicate a significant proportion of nAChRs are located at the presynaptic terminal (section 1.4), it also indicates that a significant proportion of those receptors are not found in SJCs. Metabotropic presynaptic receptors are thought to regulate the synaptic terminal and influence release (section 1.5) by controlling ion fluxes including calcium flow (Miller, 1990). In many cases the role of LGICs is less clear (Papke, 1993). Some nAChRs are permeable to calcium (section 1.3) and are inwardly rectifying, it has been suggested that these nAChRs function as a "and not" logic gate (review Papke, 1993), allowing Ca^{2+} flux when the cell is stimulated by ACh and not depolarized by other inputs. It is also possible that calcium permeable nAChRs induce calcium mediated responses in the same manor as metabotropic presynaptic receptors. To date calcium permeability has not been reported for the $\alpha 4\beta 2$. The $\alpha 4\beta 2$ isoform is rapidly desensitized (section 1.3, 1.5) and the presynaptic $\alpha 4\beta 2$ is certainly involved in transiently increasing the probability of neurotransmitter release (section 1.3), but no other function seems to have been definitively demonstrated.

References

Abood L.G., Reynolds D.T., Bidlack J.M., (1980) "Stereospecific ^{3}H-nicotine binding to intact and solubilized rat brain membranes and evidence for its noncholinergic nature" Life Sci. **27**:1307-1314

Abrams T.W., Karl K.A., Kandel E.R., (1991) "Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: dual regulation of adenylate cyclase by Ca^{2+}/calmodulin and transmitter" J. Neurosci **11**:2655-2665

Adam-Vizi V., (1992) "External Ca^{2+}-independent release of neurotransmitters" J. Neurochem. **58**:395-405

Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J.D., (1983) "Molecular biology of the cell" Garland Publishing: New York and London

Almers W. and Tse F.W., (1990) "Transmitter release from synapses:does a preassembled fusion pore initiate exocytosis?" Neuron **4**:813-818

Amar M., Thomas P., Johnson C., Lunt G.G., Wonnacott S., (1993) "Agonist pharmacology of the neuronal α7 nicotinic receptor expressed in *Xenopus* oocytes" FEBS Lett. **327**:284-288

Anand R., Conroy W.G., Schoepfer R., Whiting P., Lindstrom J., (1991)

"Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure" J. Biol. Chem. **266**:11192-11198

Aracava Y., Deshpande S.S., Swanson K.L., Rapoport H., Wonnacott S., Lunt G.G., Albuquerque E.X., "Nicotinic acetylcholine receptors in cultured neurons from the hippocampus and brain stem of the rat characterized by single channel recording" FEBS Lett. **222**:63-70

Armstrong C.M., (1992) "Voltage-dependent ion channels and their gating" Physiol Rev. (Supplement) **72**:S5-S13

Aubert I., Araujo D.M., Cécyre D., Robitaille Y., Gauthier S., Quirion R., (1992) "Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's Diseases" J. Neurochem. **58**:529-541

Balfour D.J.K. (1982) "The effects of nicotine on brain neurotransmitter systems" Pharmacol. Ther. **16**:269-282

Ballivet M., Patrick J., Lee J., Heinemann S., (1982) "Molecular cloning of cDNA coding for the gamma subunit of *Torpedo* acetylcholine receptor" Proc. Natl. Acad. Sci. USA **79**:4466-4470

Ballivet M., Nef P., Couturier S., Rungger D., Bader C.R., Bertrand D., Cooper E., (1988) "Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes after cDNA injection" Neuron **1**:847-852

Barnes K., Turner A.J., Kenny A.J., (1988) "Electronmicroscopic immunocytochemistry of pig brain shows that endopeptidase-24.11 is localized in neuronal membranes" Neurosci. Lett. **94**:64-69

Barnes K., Turner A.J., Kenny A.J., (1992) "Membrane localization of endopeptidase-24.11 and peptidyl dipeptidase A (angiotensin converting enzyme) in the pig brain: a study using subcellular fractionation and electron immunocytochemistry" J. Neurochem. **58**:2088-2096

Baude A., Nusser Z., Roberts J.D.B., Mulvihill E., McIlhinney R.A.J., Somogyi P., (1993) "The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction" Neuron **11**:771-787

Beesley, J.E., (1989) "Colloidal gold: a new perspective for cytochemical marking" Royal Microscopical Society, Microscopy Handbooks 17, Oxford University Press: Oxford

Bendayan, M., and Zollinger, M., (1982) "Protein A immunocytochemical techniques: ultrastructural localisation of antigens on post-osmicated tissues" Proc. 10th Int. Congr. Electron Microsc., **3**:267-268

Bennet M.V.L., Barrio L.C., Bargiello T.A., Spray D.C., Hertzerg E., Sáez J.C., (1991) "Gap junctions:-new tools, new answers, new questions", Neuron **6**:305-320

Benowitz N.L.,(1988) "Pharmacological aspects of cigarette smoking and nicotine addiction" N. Engl. J. Med. **319**:1318-1330

Benwell M.E.M.,Balfour D.J.K.,(1985a) "Central nicotine binding sites: a study of post-mortem stability" Neuropharmacology **24**:1135-1137

Benwell M.E.M.,Balfour D.J.K.,(1985b) "Nicotine binding to brain tissue from drug-naive and nicotine-treated rats" J. Pharm. Pharmacol. **37**:405-409

Benwell M.E.M.,Balfour D.J.K.,Anderson J.M.,(1988) "Evidence that tobacco smoking increases the density of (-)-[^{3}H]nicotine binding-sites in human brain" J. Neurochem. **50**:1243-1247

Bertrand D., Devillers-Thiéry A., Revah F., Galzi J.-L., Hussy N., Mulle C., Bertrand S.,Ballivet M.,Changeux J.-P.(1992) "Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain" Proc. Natl. Acad. Sci. USA **89**:1261-1265

Bertrand D., Valera S., Bertrand S., Ballivet M., Rungger D., (1991) "Steroids inhibit nicotinic acetylcholine receptors" Neuroreport **2**:277-280

Betz H., (1992) "Structure and function of inhibitory glycine receptors" Q. Rev. Biophys. **25**:381-394

Bock G., Marsh J., eds. (1990) "The biology of nicotine dependence". Ciba

Foundation Symposium 152. Wiley: Chichester

Bossy B., Ballivet M., Spierer P., (1988) "Conservation of neural nicotinic acetylcholine receptors from *Drosophila* to vertebrate central nervous systems" EMBO J. **7**:611-618

Boulter J., Connolly J., Deneris E., Goldman D., Heinemann S., Patrick J., (1987) "Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family" Proc. Natl. Acad. Sci. USA **84**:7763-7767

Bourne H.R., Nicoll R., (1993) "Molecular Machines integrate coincident synaptic signals" Cell **72**/Neuron **10**:65-76

Bradford H.F., Jones D.G., Ward H.K., Booher J., (1975) "Biochemical and morphological studies of the short and long term survival of isolated nerve-endings" Brain Res. **90**:245-259

Bruns R.F., Lawson-Wendling K., Pugsley T.A., (1983) "A rapid filtration assay for soluble receptors using polyethylenimine-treated filters" Anal. Biochem. **132**:74-81

Burdett I.D.J., Rogers H.J., (1970) "Modifications of the appearance of mesosomes in sections of *Bacillus licheniformis* according to the fixation procedures" J. Ultrastruct. Res. **30**:354-367

Burry R.W., Vandre' D.D., Hayes D.M., (1992) "Silver enhancement of gold antibody probes in pre-embedding electron microscopic immunocytochemistry" J. Histochem. Cytochem. **40**:1849-1856

Cachelin A.B., Jaggi R., (1991) "ß subunits determine the time course of desensitization in rat α3 neuronal nicotinic acetylcholine receptors" Pflügers Arch. **419**:579-582

Catterall W.A.,(1992) "Cellular and molecular biology of voltage-gated sodium channels", Physiol. Rev. (supplement) **72**:S15-S48

Chang, C.H., Beer, M. and Marzilli L.G. (1977) "Osmium-labelled polynucleotides. The reaction of osmium tetroxide with deoxyribonucleic acid and synthetic polynucleotides in the presence of tertiary nitrogen donor ligands" Biochemistry **16**:33-38

Changeux J. -P., (1981) "The acetylcholine receptor: an allosteric membrane protein" Harvey Lect. **75**:85-254

Changeux J.-P.,(1983) translated Garey L.,(1985) "Neuronal Man. The biology of mind" Oxford University Press: New York

Changeux J.-P., Devillers-Thie'ry A., Galzi J.-L., Bertrand D., (1992a) "New mutants to explore nicotinic receptor functions" Trends Pharmacol. Sci. **13**:299-301

Changeux J.-P., Galzi J.-L., Devillers-Thie'ry A., Bertrand D., (1992b) "The functional architecture of the acetylcholine nicotinic receptor explored by affinity labelling and site-directed mutagenesis" Q. Rev. Biophys. **25**:395-432

Changeux J.-P.,Revah F.,(1987) "The acetylcholine receptor molecule: allosteric sites and the ion channel" Trends NeuroSci. **10**:245-250

Chaplin A.J., (1985) "Tannic acid histology: a historical perspective" Stain Technol. **60**:219-231

Charnet P., Labarca C., Leonard R.J., Vogelaar N.J., Czyzyk L., Gouin A., Davidson N., Lester H.A., (1990) "An open-channel blocker interacts with adjacent turns of α-helices in the nicotinic acetylcholine receptor" Neuron **4**:87-95

Clarke P.B.S.,(1987) "Nicotine and smoking: a perspective from animal studies" Psychopharmacology **92**:135-143

Clarke P.B.S.,(1991) "Nicotinic receptor blockade therapy and smoking cessation" Br. J. Addict. **86**:501-505

Clarke P.B.S., (1992) "The fall and rise of neuronal α-bungarotoxin binding proteins" Trends Pharmacol. Sci. **13**:407-413

Clarke P.B.S., Fu D.S., Jakubovic A., Fibiger H.C., (1988) "Evidence that mesolimbic dopaminergic activation underlies the locomotor stimulant action of

nicotine in rats" J. Pharmacol. Exp. Ther. **246**:701-708

Clarke P.B.S., Hamill G.S., Nadi N.S., Jacobwitz D.M., Pert A., (1986) "^{3}H-nicotine and ^{125}I-α-bungarotoxin-labelled nicotinic receptors in the interpeduncular nucleus of rats. II Effects of habenular deafferentation" J. Comp. Neurol. **251**:407-413

Clarke P.B.S.,Pert A., (1985) "Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons" Brain Res. **348**:355-358

Clarke P.B.S., Schwartz R.D., Paul S.M., Pert C.B., Pert A., (1985) "Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]-acetylcholine, [^{3}H]-nicotine, and [^{125}I]-α-bungarotoxin" J. Neurosci. **5**:1307-1315

Claudio T., Ballivet M., Patrick J., Heinemann S., (1983) "Nucleotide and deduced amino acid sequences of *Torpedo californica* acetylcholine receptor gamma-subunit" Proc. Natl. Acad. Sci USA **80**:1111-1115

Cockcroft V.B., Osguthorpe D.J., Barnard E.A., Friday A.F., Lunt G.G., (1992) "Ligand-gated ion-channels: homology and diversity" Mol. Neurobiol. **4**:129-169

Cohen M.W.,Jones O.T.,Angelides K.J.,(1991) "Distribution of Ca^{2+} channels on frog motor nerve terminals revealed by fluorescent ω-conotoxin" J. Neurosci. **11**:1032-1039

Connolly J.G., (1989) "Mini-review. Structure-function relationships in nicotinic acetylcholine receptors" Comp. Biochem. Physiol. **93A**:221-231

Connolly J., Boulter J., Heinemann S.F., (1992) "α4-2ß2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs" Br. J. Pharmacol. **105**:657-666

Conroy W.G., Vernallis A.B., Berg D.K., (1992) "The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain" Neuron **9**:679-691

Conti-Tronconi B.M., Raftery M.A., (1982) "The nicotinic cholinergic receptor: correlation of molecular structure with functional properties" Annu. Rev. Biochem. **51**:491-530

Cooper E., Couturier S., Ballivet M., (1991) "Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor" Nature (London) **350**:235-238

Cotman C.W., Taylor D., (1972) "Isolation and structural studies on synaptic complexes from rat brain" J. Cell Biol. **55**:696-711

Couturier S., Bertrand D., Matter J.-M., Hernandez M.-C., Bertrand S., Millar N., Valera S., Barkas T., Ballivet M., (1990a) "A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric

channel blocked by αBtx" Neuron **5**:847-856

Couturier S., Erkman L., Valera S., Rungger D., Bertrand S., Boulter J., Ballivet M., Bertrand D., (1990b) "α5, α3, and non-α3: three clustered avian genes encoding neuronal nicotinic acetylcholine receptor-related subunits" J. Biol. Chem. **265**:17560-17567

Dale, H.H., (1935) "Pharmacology and nerve endings" Proc. R. Soc. Med. **28**:319-332

Daubas P., Devillers-Thiéry A., Geoffroy B., Martinez S., Bessis A., Changeux J.-P., (1990) "Differential expression of the neuronal acetylcholine receptor α2 subunit gene during chick brain development" Neuron **5**:49-60

De Blas A.L., Cherwinski H.M., (1983) "Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies" Anal. Biochem. **133**:214-219

Dechesne C.J., Oberdorfer M.D., Hampson D.R., Wheaton K.D., Nazarali A.J., Goping G., Wenthold R.J., (1990) "Distribution of a putative kainic acid receptor in the frog central nervous system determined with monoclonal and polyclonal antibodies: Evidence for synaptic and extrasynaptic localization" J. Neurosci. **10**:479-490

Del Castillo J., Katz B. (1954) "Quantal components of the end-plate potential" J. Physiol. (London), **124**:560-573

DeLorme E.M., McGee R. (1988) "Effect of prolonged depolarization on the nicotinic acetylcholine receptor of PC12 cells" J. Neurochem. **50**:1248-1252

Deneris E.S., Connolly J., Boulter J., Wada E., Wada K., Swanson L.W., Patrick J., Heinemann S., (1988) "Primary structure and expression of ß2: a novel subunit of neuronal nicotinic acetylcholine receptors" Neuron **1**:45-54

Deneris E.S., Connolly J., Rogers S.W. Duvoisin R., (1991) "Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors" Trends Pharmacol. Sci. **12**:34-40

De Robertis E.D.P., Bennett H.S., (1955) "Some features of the submicroscopic morphology of synapses in frog and earthworm" J. Biophys. Biochem. Cytol. **1**:47-58

Devillers-Thiéry A., Giraudat J., Bentaboulet M., Changeux J.-P. (1983) "Complete mRNA coding sequence of the acetylcholine binding alpha subunit of *Torpedo marmorata* acetylcholine receptor: A model for the transmembrane organization of the polypeptide chain" Proc. Natl. Acad. Sci USA **80**:2067-2071

DeVries S.H., Baylor D.A. (1993) "Synaptic circuitry of the retina and olfactory bulb" Cell **72**/Neuron **10**:139-149

Deutch A.Y., Holliday J., Roth R.H., Chun. L.L.Y., Hawrot E., (1987) "Immunohistochemical localization of a neuronal nicotinic acetylcholine receptor

in mammalian brain" Proc. Natl. Acad. Sci. USA **84**:8697-8701

Docherty M., Bradford H.F., Cash C.D., Ehret M., Maitre M., Joh T.H., (1991) "Isolation of monoaminergic synaptosomes from rat brain by immunomagnetophoresis" J. Neurochem. **56**:1569-1580

Docherty M., Bradford H.F., Joh T.H., (1989) "Isolation of catecholaminergic synaptosomes from mammalian brain" Biochem. Soc. Trans. **17**:775-777

Docherty M., Bradford H.F., Wu J.-Y., (1987a) "Co-release of glutamate and aspartate from cholinergic and GABAergic synaptosomes" Nature (London) **330**:64-66

Docherty M., Bradford H.F., Wu J.-Y., (1987b) "Isolation of highly purified cholinergic and GABAergic synaptosomes from mammalian brain using an immunomagnetophoretic technique" Biochem. Soc. Trans. **15**:638-640

Drasdo A.L., (1993) "Studies of brain nicotinic acetylcholine receptors with respect to nicotine dependence" PhD thesis, Bath University

Dreher, K.D., Schulman, J.H., Anderson, O.R. and Roels, O.A. (1967) "The stability and structure of mixed lipid monolayers and bilayers. I. Properties of lipid and lipoprotein monolayers on osmium tetroxide solutions and the role of cholesterol, retinal and tocopherol in stabilizing lecithin monolayers" J. Ultrastruct. Res., **19**:586-599

Dunkley P.R., Heath J.W., Harrison S.M., Harvie P.E., Glenfield P.J., Rostas J.A.P., (1988) "A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions" Brain Res. **441**:59-71

Dunkley P.R., Rostas J.A.P., Heath J.W., Powis D.A., (1987) "The preparation and use of synaptosomes for studying secretion of catecholamines" in "The secretory Process Vol 3: in vitro methods for studying secretion" eds: Poisner A., Trifaro J.M., Elsevier:Amsterdam pp 315-334

Duvoisin R.M., Deneris E.S., Patrick J., Heinemann S., (1989) "The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: ß4" Neuron **3**:487-496

Dwyer B.P., (1988) "Evidence for the extramembranous location of the putative amphipathic helix of acetylcholine receptor" Biochemistry **27**:5586-5592

Eccles J.C., (1963) "The ionic mechanisms of postsynaptic inhibition" In Les Prix Nobel en 1963 (Stockholm: The Nobel Foundation) pp261-283

Edelman G.M., (1993) "Neural Darwinism: selection and reentrant signaling in higher brain function" Neuron **10**:115-125

Edwards F.A., Konnerth A., Sakmann B., (1990) "Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch clamp

study" J. Physiol. **430**:213-249

Erlanger B.F., Wassermann N.H., Cleveland W.L., Penn A.S., Hill B.L., Sarangarajan R., (1984) "Anti-idiotypic route to antibodies to the acetylcholine receptor and experimental myasthenia Gravis" Chapter 9 from "Monoclonal and anti-idiotypic antibodies: Probes for receptor structure and function" Eds Venter J.C., Fraser C.M., Lindstrom J., A.R.Liss: New York. pp163-176

Fatt P., Katz B. (1950) "Some observations on biological noise" Nature (London) **166**:597-598

Fatt P., Katz B., (1952) "Spontaneous subthreshold activity at motor nerve endings" J. Physiol. (London) **117**:109-128

Faulk W.P., Taylor G.M., (1971) "An immunocolloid method for the electron microscope" Immunocytochemistry **8**:1081-1083

Fleming L.H., Hodach A.E., Reynolds N.C., (1980) "Effects of isolation and incubation on the biochemical and morphological integrity of synaptosomes" Brain Res. **202**:469-473

Flores C.M., Rogers S.W., Pabreza L.A., Wolfe B.B., Kellar K.J., (1992) "A subtype of nicotinic cholinergic receptor in rat brain is composed of $\alpha 4$ and ß2 subunits and is up-regulated by chronic nicotine treatment" Mol. Pharmacol. **41**:31-37

Fluhler E.N., Lippiello P.M., Fernandes K.G., (1992) "Localization and function of high-affinity nicotine receptors on cultured cortical neurons from rat brain" Chap. 3 of "The biology of nicotine: current research issues" Eds Lippiello P.M., Collins A.C., Gray J.A., Robinson J.H., Raven Press: New York pp 23-37

Fox A.P., Nowycky M.C., Tsien R.W., (1987) "Kinetic and pharmacological properties distinguishing 3 types of calcium currents in chick sensory neurons" J. Physiol. (London) **394**:149-172

Fraser C.M., Lindstrom J., (1984) "The use of monoclonal antibodies in receptor characterization and purification" Chap. 1 of "Molecular and chemical characterization of membrane receptors" Eds Venter J.C., Harrison L.C., Alan R. Liss: New York pp1-30

Froehner S.C.,(1993) "Regulation of ion channel distribution at synapses" Annu. Rev. Neurosci. **16**:347-368

Fung Y.K.,(1989) "Effects of chronic nicotine pretreatment on (+)-amphetamine and nicotine-induced synthesis and release of ^{3}H-dopamine from ^{3}H-tryosine in rat nucleus accumbens" J. Pharm. Pharmacol. **41**:66-68

Galzi J.-L., Betrand D., Devillers-Thiéry A., Revah F., Betrand S., Changeux J,-P, (1991a) "Functional significance of aromatic amino acids from three peptide loops of the α7 neuronal nicotinic receptor site investigated by site directed mutagenesis" FEBS Lett. **294**:198-202

Galzi J.-L.,Devillers-Thiéry A.,Hussy N.,Betrand S.,Changeux J,-P,Betrand D., (1992) "Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic" Nature (London) **359**:500-505

Galzi J.-L.,Revah F.,Bessis A.,Changeux J.-P.,(1991b) "Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain" Annu. Rev. Pharmacol. **31**:37-72

Galzi J.-L.,Revah F.,Black D.,Goeldner M.,Hirth C.,Changeux J.-P.,(1990) "Identification of a novel amino acid α-tyrosine 93 within the cholinergic ligand-binding sites of the acetylcholine receptor by photoaffinity labeling: additional evidence for a three-loop model of the cholinergic ligand-binding sites" J. Biol. Chem. **265**:10430-10437

Gasic G.P., Hollmann M., (1992) "Molecular neurobiology of glutamate receptors" Annu. Rev. Physiol. **54**:507-536

Gehle V.M.,Sumikawa K.,(1991) "Site-directed mutagenesis of the conserved N-glycosylation site on the nicotinic acetylcholine receptor subunits" Mol. Brain Res. **11**:17-25

Giacobini E., (1992) "Nicotinic acetylcholine receptors in the human cortex" Chap. 12 of "The biology of nicotine: current research issues" Eds Lippiello P.M.,Collins A.C.,Gray J.A.,Robinson J.H., Raven Press: New York pp183-194

Giraudat J., Dennis M., Heidmann T., Chang J.-Y., Changeux J.-P., (1986) "Structure of the high-affinity binding site for non-competitive blockers of the acetylcholine receptor: serine-262 of the δ-subunit is labeled by [^{3}H]-chlorpromazine" Proc. Natl. Acad. Sci. USA **83**:2719-2723

Glauert A.M., (1977) "Fixation, dehydration and embedding of biological specimens" Vol. 3 Part I of "Practical methods in electron microscopy" Ed Glauert A.M., Elsevier/North-Holland Biomedical Press: Amsterdam-New York-Oxford pp95-99

Glauert A.M., Glauert R.H., (1958) "Araldite as an embedding medium for electron microscopy" J. Biophys. Biochem. Cytol., **4**:191-194

Goodman C.S., Shatz C.J., (1993) "Developmental mechanisms that generate precise patterns of neuronal connectivity" Cell **72**/Neuron **10**:77-98

Grady S., Marks M.J., Wonnacott S., Collins A.C., (1992) "Characterization of nicotinic receptor-mediated [^{3}H]dopamine release from synaptosomes prepared from mouse striatum" J. Neurochem. **59**:848-856

Gray E.G., Whittaker V.P.,(1962) "The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation" J. Anat. **96**:79-87

Griffiths G., Hoppeler H., (1986) "Quantitation in immunocytochemistry:

correlation of immunogold labelling to absolute number of membrane antigens" J. Histochem. Cytochem. **34**:1389-98

Guy H.R., Hucho F., (1987) "The ion channel of the nicotinic acetylcholine receptor" Trends NeuroSci **10**:318-321

Hagino N., Lee J.W., (1985) "Effect of maternal nicotine on the development of sites for [^{3}H] nicotine binding in the fetal brain" Int. J. Dev. Neurosci. **3**:567-571

Hake, T. (1965) "Studies on the reactions of osmium tetroxide and potassium permanganate with amino acids, peptides and proteins" Lab. Invest., **14**:1280

Hall Z.W., Sanes J.R., (1993) "Synaptic structure and development: the neuromuscular junction" Cell **72**/Neuron **10**:99-122

Harrison S.M., Jarvie P.E., Dunkley P.R. (1988) "A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: viability of subcellular fractions" Brain Res. **441**:72-80

Harsing L.G., Sershen H., Lajtha A., (1992) "Dopamine efflux from striatum after chronic nicotine: evidence for autoreceptor desensitization" J. Neurochem. **59**:48-54

Hayat, M.A. (1981) "Fixation for electron microscopy" Academic Press, San Diego and London

Hayat, M.A. (1986)a "Glutaraldehyde: role in electron microscopy" Micron. Microsc. Acta, **17**:115-135

Hayat, M.A. (1986)b "Basic Techniques for Transmission Electron microscopy" Academic Press San Diego and London

Hayat, M.A. (1989a) "Principles and Techniques of Electron Microscopy. Biological Applications" 3rd Edn MacMillian Press

Hayat M.A.,(Ed) (1989b) "Colloidal gold: principles, methods, and applications" Academic Press: San Diego & Lond.

He X., Rosenfeld M.G. (1991) "Mechanisms of complex transcriptional regulation: implications for brain development" Neuron **7**:183-196

Hebb D.O. (1949) "The organization of behaviour: a neuropsychological theory" Wiley: New York

Heidmann T., Oswald R.E., Changeux J.-P.,(1983) "Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from *Torpedo marmorata*" Biochemistry **22**:3112-3127

Heuser J.E., Salpeter S.R., (1979) "Organization of acetylcholine receptors in quick frozen, deep etched, and rotary-replicated *Torpedo* postsynaptic membrane" J. Cell Biol. **82**:150-173

Hill J.A., Zoll M., Bourgeois J.-P., Changeux J.-P., (1993) "Immunocytochemical localization of a neuronal nicotinic receptor: the ß2-subunit" J. Neurosci. **13**:1551-1568

Hillard C.J., Pounds J.J., (1993) "Effects of chronic nicotine treatment on the accumulation of [^{3}H]-tetraphenylphosphonium by cerebral cortical synaptosomes" J. Neurochem. **60**:687-695

Hille B., (1992) "Ionic Channels of Excitable Membrane" 2nd Edn. Sunderland, Massachusetts: Sinauer

Hodgkin A.L., Huxley A.F., (1952) "A quantitative description of membrane current and its application to conduction and excitation in nerve" J. Physiol. (London) **117**:500-544

Hodgkin A.L., Huxley A.F., Katz B., (1949) "Ionic currents underlying activity in the giant axon of the squid" Arch. Sci. Physiol. **3**:129-150

Holgate C.S., Jackson P., Cowen Ph.N., Bird C.C., (1983) "Immunogold-silver staining: new method of immunostaining with enhanced sensitivity" J. Histochem. Cytochem. **31**:938-944

Hollenberg M.D. (1985a) "Examples of homospecific and heterospecific receptor regulation" Trends Pharmacol. Sci. **6**:242-245

Hollenberg M.D. (1985b) "Biochemical mechanisms of receptor regulation" Trends Pharmacol. Sci. **6**:299-302

Hopwood D., (1970) "The reactions between formaldehyde, glutaraldehyde, and osmium tetroxide, and their fixation effects on bovine serum albumin and on tissue blocks" Histochemie **24**:50-64

Horisberger, M. (1981) "Colloidal gold: a cytochemical marker for light and fluorescent microscope and for transmission and scanning electron microscopy" Scanning Electron Microsc. **2**:9-32

Horisberger, M. and Rosset, J. (1977) "Colloidal gold, a useful marker for transmission and scanning electron microscopy" J. Histochem. Cytochem. **25**:295-305

Hucho F., Oberthu¨r W., Lottspeich F., (1986) "The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices MII of the receptor subunits" FEBS Lett. **205**:137-142

Huganir R.L., Delcour A.H., Greengard P., Hess G., (1986) "Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization" Nature (London) **321**:774-776

Huganir R.L., Greengard P., (1990) "Regulation of neurotransmitter desensitization by protein phosporylation" Neuron **5**:555-567

Hulihan-Giblin B.A., Lumpkin M.D., Kellar K.J., (1990) "Effects of chronic administration of nicotine on prolactin release in the rat: inactivation of prolactin response by repeated injections of nicotine" J. Pharmacol. Exp. Ther. **252**:21-25

Imoto K., Busch C., Sakmann B., Mishina M., Konno T., Nakai T., Bujo H., Mori Y., Fukunda K., Numa S., (1988) "Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance" Nature (London) **335**:645-648

Imoto K., Methfessel C., Sakmann B., Mishina M., Mori Y., Konno T., Fukuda K., Kurasaki M., Bujo H., Fujita Y., Numa S., (1986) "Location of δ-subunit region determining ion transport through the acetylcholine receptor channel" Nature (London) **324**:670-674

Irons J., Lotwick H.S., Lunt G.G., Wonnacott S., Whiting P., Lindstrom J.M., (1989) "Isolation of a synaptosomal subpopulation bearing [^{3}H]-nicotine receptors by an immunoaffinity technique" Biochem. Soc. Trans. **17**:773

Irons J., Lunt G.G., Wonnacott S., Whiting P., Lindstrom J., (1988) "Immunoaffinity isolation of hippocampal nerve terminals bearing [^{3}H]nicotine receptors" Abs. Soc. Neurosci. 18th annual meeting, 440.3

Iversen L.L., (1979) "The chemistry of the brain" Sci. Am. **241(3)**:118-129

James T.H., (1977) "The theory of the photographic process" Macmillan: New

York

Jennings K.R., Brown D.G., Wright D.P., (1986) "Methyllcaconitine, a naturally - occuring insecticide with high-affinity for the insect cholinergic receptor" Experientia **42**:611-613

Jessel T.M., Kandel E.R., (1993) "Synaptic Transmission: a bidirectional and self-modifiable form of cell-cell communication" Cell **72**/Neuron **10**:1-30

Johnson M.K., (1960) "The intracellular distribution of glycolytic and other enzymes in rat brain homogenates and brain mitochondrial preparations" Biochem. J., **77**:610-618

Johnson M.K., Whittaker V.P., (1963) "Lactate dehydrogenase as a cytoplasmic marker in brain" Biochem. J. **88**:404-409

Jones D.G., (1975) "Synapses and synaptosomes. Morphological aspects" Chapman and Hall: London

Kao P.N., Dwork A.J., Kandaney R.J., Silver M.L., Wideman J., Stein S., Karlin A., (1984) "Identification of two alpha-subunit half-cysteins specifically labelled by an affinity reagent for the acetylcholine binding site" J. Biol. Chem. **259**:1162-1165

Karlin A., (1990) "Explorations of the nicotinic acetylcholine receptor" Harvey

Lect. **85**:71-107

Karnovsky M.J., (1965) "A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy" J. Cell Biol., **27**:137, Abs 270

Katz B., (1969) "The release of neural transmitter substances" Springfield, Illinois: C.C. Thomas

Katz B., Thesleff S., (1957) "A study of the 'desensitization' produced by acetylcholine at the motor end-plate" J. Physiol. **138**:63-80

Kellar K.J., Wonnacott S., (1990) "Nicotinic cholinergic receptors in Alzheimer's disease" Chap. 10 of "Nicotine psychopharmacology: molecular, cellular and behavioural aspects" Eds Wonnacott S., Russel M.A.H., Stollerman I.P., Oxford University Press: New York pp341-373

Kelly R.B., (1993) "Storage and release of neurotransmitters" Cell **72**/Neuron **10**:43-54

Kirkeby S., Moe D., (1986) "Studies on the actions of glutaraldehyde, formaldehyde, and mixtures of glutaraldehyde and formaldehyde on tissue proteins" Acta Histochem. **79**:115-121

Koelle G.B., Davis R., Smyri E.G., Fine A.V., (1974) "Refinement of the bis-(thioacetoxy)aurate(I) method for the electron microscopic localization of

acetylcholinesterase and nonspecific cholinesterase" J. Histochem. Cytochem. **22**:252-259

Konno T., Busch C., von Kitzing E., Imoto K., Wang F., Nakai J., Mishina M., Numa S., Sakmann B., (1991) "Rings of anionic amino acids as structural determinants of ion selectivity in the acetylcholine receptor channel" Proc. R. Soc. Lond. (B) **244**:69-79

Ksir C., Hakan R.L. Kellar K.J., (1987) "Chronic nicotine and locomotor-activity-influences of exposure dose and test dose" Psychopharmacology **92**:25-29

Kuhse J., Laube B., Magalei D., Betz H., (1993) "Assembly of the inhibitory glycine receptor: identification of amino acid sequence motifs governing subunit stoichiometry" Neuron **11**:1049-1056

Kyte J., Doolittle R.F., (1982) "A simple method for displaying the hydrophobic character of a protein " J. Mol. Biol. **157**:105-132

Lackie P.M., Hennessy R.J., Hacker G.W., Polak J.M., (1985) "Investigation of immunogold-silver staining by electron microscopy" Histochemistry **83**:545-550

Laemmli U.K., (1970) "Cleavage of structural proteins during the assembly of the head of the bacteriophage T4" Nature (London) **227**:680-685

Landis S.C., (1980) "Developmental changes in the neurotransmitter properties

of dissociated sympathetic neurons: a cytochemical study of the effects of medium" Dev. Biol. **77**:349-361

Lange K.W., Wells F.R., Rossor M.N., Jenner P., Marsden C.D., (1991) "Cortical nicotinic receptors in Alzheimers-disease and Parkinsons-disease" J. Neurol. Neurosurg. Psychiatry **54**:373-374

Lapchak P.A., Araujo D.M., Quirion R., Collier B., (1989) "Effect of chronic nicotine treatment on nicotinic autoreceptor function and N-[^{3}H]methylcarbamylcholine binding sites in the rat brain" J. Neurochem. **52**:483-491

Larkman A., Stratford K., Jack J., (1991) "Quantal analysis of excitatory synaptic action and depression in hippocampal slices" Nature (London) **350**:344-347

La Rochelle W.J., Wray B.E., Sealock R., Froehner S.C., (1985) "Immunochemical demonstration that aminoacids 360-377 of the acetylcholine receptor gamma-subunit are cytoplasmic" J. Cell. Biol. **100**:684-691

Latta H., Hartmann J.F., (1950) "Use of a glass edge in thin sectioning for electron microscopy" Proc. Soc. Exp. Biol. Med. **74**:436-439

Lee C.Y., (1979) "Recent advances in chemistry and pharmacology of snake toxins" In "Advances in cytopharmacology" Eds. Ceccarelli B., Clementi F., Raven Press: New York Vol.3

Lenard J., Singer S.J., (1968) "Alterations of the conformation of proteins in red blood cell membranes and in solution by fixatives used in electron microscopy" J. Cell Biol. **37**:117-121

Lentz T.L., Chester J., (1977) "Localization of acetylcholine receptors in central synapses" J. Cell Biol. **75**:258-267

Leonard R.J., Labarca C.G., Charnet P., Davidson N., Lester H.A., (1988) "Evidence that the M2 memnbrane-spanning region lines the ion channel pore of the nicotinic receptor" Science **242**:1578-1581

Lewis P.R., Knight D.P., (1977) "Staining methods for sectioned material", Vol. 5 Part I of "Practical methods in electron microscopy" Ed Glauert A.M., North-Holland: New York

Lim N.F., Nowycky M.C., Bookman R.J., (1990) "Direct measurement of exocytosis and calcium currents in single vertebrate nerve terminals" Nature (London) **344**:449-451

Lindau M., Stuenkel E.L., Nordmann J.J., (1992) "Depolarization, intracellular calcium and exocytosis in single vertebrate nerve endings" Biophys. J. **61**:19-30

Lindstrom, J., (1984) "Nicotinic acetylcholine receptors: use of monoclonal antibodies to study synathesis, structure, function and autoimmune response" Chapter 2 of "Monoclonal and anti-idiotypic antibodies: Probes for receptor

structure and function" Eds Venter J.C., Fraser C.M., Lindstrom J., A.R.Liss: New York. pp21-57

Lindstrom J., (1985) "Techniques for studying the biochemistry and cell biology of receptors" from Yamamura *et al*, 1985 pp123-152

Lindstrom J., (1986) "Probing nicotinic acetylcholine receptors with monoclonal antibodies" Trends Neuro. Sci. **9**:401-407

Lindstrom J., Einarson B., Tzartos S., (1981) "Production and assay of antibodies to acetylcholine receptors" Chap. 29 Methods in Enzymology **74**:432-460 Academic Press: New York

Lindstrom J., Schoepfer R., Whiting P., (1987) "Molecular studies of the neuronal nicotinic acetylcholine receptor family" Mol. Neurobiol. **1**:281-337

Lindstrom J., Schoepfer R., Whiting P., Anand R., Conroy W.G., Saedi S., Das M., (1991) "Molecular antibody probes for nicotinic receptors of muscle and nerves" Biochem. Soc. Trans **19**:115-120

Lippiello P.M., Collins A.C., Gray J.A., Robinson J.H., eds. (1992) "The biology of nicotine:current research issues" Raven Press: New York

Lippiello P.M., Fernandes K.G., (1986) "The binding of L-[^{3}H] nicotine to a single class of high affinity sites in rat brain membranes" Mol. Pharmacol. **29**:448-454

Lippiello P.M., Fernandes K.G., (1988) "Identification of putative high affinity nicotinic receptors on cultured neurons" J. Pharmacol. Exp. Ther. **246**:409-416

Lippiello P.M., Fernandes K.G., Langone J.J., Bjercke R.J., (1991) "Characterization of nicotinic receptors on cultured cortical neurones using anti-idiotypic antibodies and ligand binding" J. Pharmacol. Exp. Ther. **257**:1216-1224

Listerud M., Brussard A.B., Devay P., Colman D.R., Role L.W., (1991) "Functional contribution of neuronal AChR subunits revealed by antisense oligonucleotides" Science **254**:1518-1521

Llinás R., Sugimori M., Silver R.B., (1992) "Microdomains of high calcium concentration in a presynaptic terminal", Science **256**:677-679

Lo D.C.,Pinkham J.L.,Stevens C.F.,(1991) "Role of a key cysteine residue in the gating of the acetylcholine receptor" Neuron **6**:31-40

Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J., (1951) "Protein measurement with the folin phenol reagent" J. Biol Chem., **193**:265-275

Luetje C.W.,Patrick J.,Séguéla P.,(1990a) "Nicotine receptors in the mammalian brain" FASEB J. **4**:2753-2760

Luetje C.W.,Wada K.,Rogers S.,Abramson S.N.,Tsuji K.,Heinemann S.,Patrick J., (1990b) "Neurotoxins distinguish between different neuronal nicotinic

acetylcholine receptor subunit combinations" J. Neurochem. **55**:632-640

Lukas R.J., (1991) "Effects of chronic nicotinic ligand exposure on functional activity of nicotinic acetylcholine receptors expressed by cells of the PC12 rat pheochromocytoma or the TE671/RD human clonal line" J. Neurochem. **56**:1134-1145

Lunt G.G., (1987) "Neuroreceptors" Chap.5 "Neurochemistry: a practical approach" (Eds) Turner A.J., Bachelard H.S. IRL Press: Oxford pp137-160

MacAllan D.R.E., Lunt G.G., Wonnacott S., Swanson K.L., Rapoport H., Albuquerque E.X., (1988) "Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems" FEBS Lett. **226**:357-363

Maelicke A., Plümer-Wilk R., Fels G., Spencer S.R., Engelhard M., Veltel D., Conti-Tronconi B.M., (1989) "Epitope mapping employing antibodies raised against short synthetic peptides: a study of the nicotinic acetylcholine receptor" Biochemistry **28**:1396-1405

Makowski L.,Caspar D.L.D.,Phillips W.C.,Baker T.S.,Goodenough D.A.,(1984) "Gap junction structures. VI. Variation and conservation in connexion conformation and packing" Biophys. J. **45**:208-218

Makowski L., Caspar D.L.D., Phillips W.C., Goodenough D.A., (1977) "Gap

junction structures. II. Analysis of the X-ray diffraction data" J. Cell. Biol. **74**:629-645

Marchbanks R.M., (1967) "Osmotically sensitive potassium and sodium compartments of synaptosomes" Biochem. J. **104**:148-157

Margiotta J.F.,Berg D.K.,(1986) "Enkephalin and substance P Modulate synaptic properties of chick ciliary ganglion neurons in cell culture" Neuroscience **18**:175-182

Marks M.J.,Burch J.B.,Collins A.C.,(1983) "Effects of chronic nicotine infusion on tolerance development and nicotinic receptors" J. Pharmacol. Exp. Ther. **226**:817-825

Marks M.J., Romm E., Gaffney D.K., Collins A.C., (1986) "Nicotine-induced tolerance and receptor changes in four mouse strains" J. Pharmacol. Exp. Ther. **237**:809-819

Marks M.J.,Stitzel J.A.,Collins A.C.,(1985) "Time course study of the effects of chronic nicotine infusion on drug response and brain receptors" J. Pharmacol. Exp. Ther. **235**:619-628

Markwell M.A.K.,Hass S.M.,Bieber L.L.,Tolbert N.E.,(1978) "A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples" Anal. Biochem. **87**:206-210

Marshall A.T.,(1980) "Frozen-hydrated bulk specimens" In: "X-ray microanalysis in biology" Ed Hayat M.A., University Park Press: Baltimore

Martino-Barrows A.M.,Kellar K.J.,(1987) "[^{3}H]-acetylcholine and [^{3}H](-)-nicotine label the same recognition site in rat brain" Mol. Pharmacol. **31**:169-174

Matsas R., Kenny A.J., Turner A.J., (1986) "An immunohistochemical study of endopeptidase-24.11 ('enkephalinase') in the pig nervous system" Neuroscience **18**:991-1012

McCrea P.D.,Popot J.-L.,Engelman D.M.,(1987) "Transmembrane topography of the nicotinic acetylcholine receptor δ subunit" EMBO J. **6**:3619-3626

McGeer P.L.,Eccles J.C.,McGeer E.G. (1987) "Molecular Neurobiology of the mamalian human brain" Plenum: New York

McMahon H.T., Nicholls D.G., (1991) "The bioenergetics of neurotransmitter release" Biochim. Biophys. Acta **1059**:243-264

Miller R.J., (1990) "Receptor-mediated regulation of calcium channels and neurotransmitter release" FASEB J. **4**:3291-3299

Morel F.M.M.,Baker R.F.,Wayland H.,(1971) "Quantitation of human red blood cell fixation by glutaraldehyde" J. Cell Biol. **48**:91-100

Morris B.J., Hicks A.A., Wisden W., Darlinson M.G., Hunt S.P., Barnard E., (1990) "Distinct regional expression of nicotinic acetylcholine receptor genes in chick brain" Mol. Brain Res. 7:305-315

Mulle C., Changeux J.-P., (1990) "A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp techniques" J. Neurosci. **10**:169-175

Mulle C., Choquet D., Korn H., Changeux J.-P., (1992a) "Calcium influx through the nicotinic receptor in rat central neurons: its relevance to cellular regulation" Neuron **8**:135-143

Mulle C., Léna C., Changeux J.-P., (1992b) "Potentiation of nicotinic receptor response by external calcium in rat cetral neurons" Neuron **8**:937-945

Nakayama H., Nakashima T., Kurogochi Y., (1991) "α4 is a major acetylcholine binding subunit of cholinergic ligand affinity-purified nicotinic acetylcholine receptor from rat brains" Neurosci. Lett. **121**:122-124

Neher E., (1992) "Ion channels for communication between and within cells" Neuron **8**:605-612

Neher E., Sakmann B., (1976) "Single-channel currents recorded from membrane of denervated frog muscle fibres" Nature (London) **260**:799-802

Neher E., Sakmann B., Steinbach J.H., (1978) "The extracellular patch clamp: a method for resolving currents through individual open channels in biological membranes" Pflügers Arch. **375**:219-228

Newby A.C., (1984) "The solubilization of membrane proteins" Chap.5 "Brain receptor methodologies" Part A Eds Marangos P.J., Campbell I.C., Cohen R.M., Academic Press: Orlando pp75-93

Newman G.R., (1987) "Use and abuse of LR White" Histochem. J. **19**:118-120

Newman G.R., (1989) "LR White embedding medium for colloidal gold methods" in Hayat (1989b)

Newman G.R., Jansani B., Williams E.D., (1983) "A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope" Histochem. J., **15**:543-555

Noda M., Takahashi H., Tanabe T., Toyosato M., Furutani Y., Hirose T., Asai M., Inayama S., Miyata T., Numa S., (1982) "Primary structure of the alpha subunit precursor of the *Torpedo californica* acetylcholine receptor deduced from a cDNA sequence" Nature (London) **299**:793-797

Noda M., Takahashi H., Tanabe T., Toyosato M., Kikyotani S., Hirose T., Asai M., Takashima H., Inayama S., Miyata T., Numa S., (1983a) "Primary structures of beta- and delta- subunit precursor of *Torpedo californica* acetylcholine receptor

deduced from cDNA sequence" Nature (London) **301**:251-255

Noda M., Takahashi H., Tanabe T., Toyosato M., Kikyotani S., Furutani Y., Hirose T., Takashima H., Inayama S., Miyata T., Numa S., (1983b) "Structural homology of *Torpedo californica* acetylcholine receptor subunits" Nature (London) **302**:528-532

Nomoto H., Takahashi N., Nagaki Y., Endo S., Arata Y., Hayashi K., (1986) "Carbohydrate structures of acetylcholine receptor from *Torpedo californica*, and distribution of oligosaccharides among the subunits" Eur. J. Biochem. **157**:233-242

Norman R.I., Mehraban F., Barnard E.A., Dolly J.O. (1982) "Nicotinic acetylcholine receptor from chick optic lobe" Proc. Natl. Acad. Sci USA **79**:1321-1325

Nowycky M.C., Fox A.P., Tsien R.W., (1985) "3 types of neuronal calcium channel with different agonist sensitivity" Nature (London) **316**:440-443

O'Donohue T.L., Millington W.R., Handelmann G.E., Contreras P.C., Chronwall B.M., (1985) "On the 50th anniversity of Dale's Law: multiple neurotransmitter neurons" Trends Pharmacol. Sci. **8**:305-308

Ortells M.O., Lunt G.G. (1993) "The transmembrane region of the nicotinic receptor: is it an all-helix bundle?" Receptors and Channels *in press*

Pabreza L.A., Dhawan S., Kellar K.J., (1991) "[^{3}H]-Cytisine binding to nicotinic cholinergic receptors in brain" Mol. Pharmacol. **39**:9-12

Papke R.L.,(1993) "The kinetic properties of neuronal nicotinic receptors: genetic basis of functional diversity" Prog. Neurobiol. **41**:509-531

Papke R.L., Boulter J., Patrick J., Heinemann S., (1989) "Single-channel currents of rat neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes" Neuron **3**:589-596

Papke R.L., Heinemann S.F., (1991) "The role of the ß4-subunit in determining the kinetic properties of rat neuronal nicotinic acetylcholine α3-receptors" J. Physiol. **440**:95-112

Patterson P.H., Nawa H., (1993) "Neuronal differentiation factors/cytokines and synaptic plasticity" Cell **72**/Neuron **10**:123-138

Pauly J.R., Marks M.J., Gross S.D., Collins A.C., (1991) "An autoradiographic analysis of cholinergic receptors in mouse-brain after chronic nicotine treatment" J. Pharmacol. Exp. Ther. **258**:1127-1136

Peachey L.D., (1958) "Thin sections. I. A study of section thickness and physical distortion produced during microtomy" J. Biophys. Biochem. Cytol. **4**:233-242

Pedersen S.E., Sharp S.D., Liu W.-S., Cohen J.B., (1992) "Structure of the

noncompetitive antagonist-binding site of the *Torpedo* nicotinic acetylcholine receptor: [^{3}H]-meproadifen mustard reacts selectively with α-subunit Glu-262" J. Biol. Chem. **267**:10489-10499

Peers S.H., Smillie F., Elderfield A.J., Flower R.J., (1993) "Glucocorticoid- and non glucocorticoid induction of lipocortins (annexins), 1 and 2 in rat peritoneal leucocytes *in vivo*" Br. J. Pharmacol. **108**:66-72

Peng Y.-W., Sharp A.H., Snyder S.H., Yau K.-W (1991) "Localization of the inositol 1,4,5-triphosphate receptor in synaptic terminals in the vertebrate retina" Neuron **6**:525-531

Perä nen J., Rikkonen M., Kääriäinen L., (1993) "A method for exposing hidden antigenic sites in paraformaldehyde-fixed cultured cells, applied to initially unreactive antibodies" J. Histochem. Cytochem. **41**:447-454

Perry E.K., Perry R.H., Smith C.J., Dick D.J., Candy J.M., Edwardson J.A., Fairbairn A., Blessed G., (1987) "Nicotine receptor abnormalities in Alzheimer's and Parkinson's diseases" J. Neurol. Neurosurg. Psychiatry **50**:806-809

Polak J.M., Van Noorden S., (1984) "An introduction to immunocytochemistry: current techniques and problems" Royal Microscopical Society, Microscopy Handbooks 11, Oxford University Press: New York

Pommerleau O.F., (1984) "Neuroregulators and the reinforcement of smoking:

towards a behavioural explanation" Neurosci. Biobehav. Rev. **8**:503-513

Popot J.-L.,Changeux J.-P.,(1984) "Nicotinic receptor of acetylcholine: structure of an oligomeric integral membrane protein" Physiol. Rev. **64**:1162-1239

Pow D.V.,Morris J.F.,(1989) "Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis" Neuroscience **32**:435-439

Priestley J.V., (1987) "Immunocytochemical techniques for the localization of neurochemically characterized nerve pathways" Chap.3 "Neurochemistry: a practical approach" (Eds) Turner A.J.,Bachelard H.S. IRL Press: Oxford pp65-112

Raftery M.,Hunkapillar M.,Strader C.,Hood L.,(1980) "Acetylcholine receptor: complex of homologous subunits" Science **208**:1454-1457

Rao A., Steward O., (1991) "Evidence that protein constituents of postsynaptic membrane specializations are locally synathesized: analysis of proteins synthesized within synaptosomes" J. Neurosci. **11**:2881-2895

Rapier C., Lunt G.G., Wonnacott S., (1988) "Stereoselective nicotine-induced release of dopamine from striatal synaptosomes: concentration dependence and repetitive stimulation" J. Neurochem., **50**:1123-1130

Rapier C., Lunt G.G., Wonnacott S., (1990) "Nicotinic modulation of [^{3}H]-

Dopamine release from striatal synaptosomes: pharmacological characterization" J. Neurochem. **54**:937-945

Ratnam M., Nguyen D.L., Rivier J., Sargent P.B., Lindstrom J., (1986a) "Transmembrane Topography of nicotinic acetylcholine receptor: immunochemical tests contradict theoretical predictions based on hydrophobicity profiles" Biochemistry **25**:2633-2643

Ratnam M., Sargent P.B., Sarin V., Fox J.L., Nguyen D.L., Rivier J., Criado M., Lindstrom J., (1986b) "Location of antigenic determinants on primary sequences of subunits of nicotinic acetylcholine receptor by peptide mapping" Biochemistry **25**:2621-2632

Reichardt L.F., Tomaselli K.J., (1991) "Extracellular matrix molecules and their receptors: Functions in neuronal development" Annu. Rev. Neurosci. **14**:531-570

Reid N., (1974) "Ultramicrotomy" Vol. 3 Part II of "Practical methods in electron microscopy" Ed Glauert A.M., North-Holland: Amsterdam

Reith A., Kraemer M., Vassy J., (1984) "The influence of mode of fixation, type of fixative and vehicles on the same rat liver: a morphometric/stereologic study by light and electron microscopy" Scanning Electron Microsc. **2**:645-651

Renart J., Reiser J., Stark G., (1979) "Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying

antibody specificity and antigen structure" Proc. Natl. Acad. Sci USA **76**:3116-3120

Revah F., Bertrand D., Galzi J.-L., Devillers-Thiéry A., Mulle C., Hussy N., Bertrand S., Ballivet M., Changeux J.-P., (1991) "Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor" Nature (London) **353**:846-849

Revah F., Galzi J.-L., Giraudat J., Haumont P.-Y., Lederer F., Changeux J.-P., (1990) "The non-competitive blocker [^{3}H]-chlorpromazine labels three amino acids of the acetylcholine receptor γ subunit: implications for the α-helical organization of regions M2 and for the ion channel" Proc. Natl. Acad. Sci. USA **87**:4675-4679

Reynolds, E.S. (1963) "The use of lead citrate at high pH as electron opaque stain in electron microscopy" J. Cell Biol., **17**:208-212

Richardson P.J., Brown S.J., Bailyes E.M., Luzio J.P., (1987) "Ectoenzymes control adenosine modulation of immunoisolated cholinergic synaptosomes" Nature (London) **327**:232-234

Richardson P.J., Luzio J.P., (1986) "Review: Immunoaffinity purification of subcellular particles and organelles" Appl. Biochem. Biotechnol. **13**:133-145

Richardson P.J., Luzio J.P., (1988) "Immunoaffinity purification of membrane fractions from mammalian cells" from "Subcellular Biochemistry" Vol. 12 "Immunological aspects", Chap. 6, Ed Harris J.R., Plenum, pp221-241

Richardson P.J., Siddle K., Luzio J.P., (1984) "Immunoaffinity purification of intact, metabolically active, cholinergic nerve terminals from mammalian brain" Biochem J. **219**:647-654

Rogers S.W.,Hughes T.E.,Hollmann M.,Gasic G.P.,Deneris E.S.,Heinmann S., (1991a) "The characterization and localization of the glutamate receptor subunit Glu R1 in the rat brain" J. Neurosci **11**:2713-2724

Rogers S.W.,Gahring L.C.,Papke R.L.,Heinemann S.,(1991b) "Identification of cultured cells expressing ligand-gated cationic channels" Protein expression and Purification **2**:108-116

Rogers S.W.,Mandelzys A.,Deneris E.S.,Cooper E.,Heinemann S.,(1992) "The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF" J. Neurosci **12**:4611-4623

Role L.W., (1992) "Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels" Curr. Opin. Neurosci. **2**:254-262

Romano C., Goldstein A., (1980) "Stereospecific nicotinic receptors in rat brain membranes" Science **210**:647-650

Romm E.,Lippiello P.M.,Marks M.J.,Collins A.C.,(1990) "Purification of L-[^{3}H]-nicotine eliminates low affinity binding" Life Sci. **46**:935-943

Ross E.M., (1989) "Signal sorting and amplification through G protein-coupled receptors" Neuron **3**:141-152

Roth B., Schwendimann B., Hughes G.J., Tzartos S.J., Barkas T., (1987) "A modified nicotinic acetylcholine receptor lacking the 'ion channel amphipathic helices'" FEBS Lett. **221**:172-178

Rowell P.P.,Winkler D.L.,(1984) "Nicotine stimulation of [^{3}H]-dopamine release by nicotine in rat nucleus accumbens" J. Neurochem **49**:1449-1454

Rowell P.P., Wonnacott S., (1990) "Evidence for functional activity of up-regulated nicotine binding sites in rat striatal synaptosomes" J. Neurochem. **55**:2105-2110

Sabatini, D.D., Bensch, K. Barrnett, R.J. (1963) "Cytochemistry and electron microscopy. The preservation of cellular structure and enzymic activity by aldehyde fixation" J. Cell Biol., **17**:19-58

Sakmann B., (1992) "Elementary steps in synaptic transmission revealed by current through single ion channels" Neuron **8**: 613-629

Sanderson E.M. (1993) "The effect of nicotine on noradrenergic transmission in the rat central nervous system" PhD thesis, Bath University

Sanderson E.M.,Drasdo A.L.,McCrea K.,Wonnacott S.,(1993) "Up-regulation

of nicotinic receptors following continuous-infusion of nicotine is brain-region-specific" Brain Res. **617**:349-352

Sargent P.B.,(1993) "The diversity of neuronal nicotinic acetylcholine receptors" Annu. Rev. Neurosci. **16**:403-443

Sargent P.B., Pike S.H., Nadel D.B., Lindstrom J.M., (1989) "Nicotinic acetylcholine receptor-like molecules in the retina, retinotectal pathway, and optic tectum of the frog" J. Neurosci **9**:565-573

Schoepfer R., Conroy W.G., Whiting P., Gore M., Lindstrom J., (1990) "Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily" Neuron **5**:35-48

Schwartz R.D., Kellar K.J., (1985) "*In vivo* regulation of [^{3}H] acetylcholine recognition sites in brain by nicotinic cholinergic drugs" J. Neurochem. **45**:427-433

Schwartz R.D., Lehman J., Kellar K.J., (1984) "Presynaptic nicotinic cholinergic receptors labelled by [^{3}H]acetylcholine on catecholamine and serotonin axons in brain" J. Neurochem. **42**:1495-1498

Schwartz R.D., McGee R., Kellar K.J., (1982) "Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain" Mol. Pharmacol. **22**:56-62

Shantz E.M., (1983) Revised and updated by Palmer E., "Pansorbin

Staphylococcus aureus cells: Review and bibliography of the immunological applications of fixed protein A-bearing *Staphylococcus aureus* cells, Cowan I strain" 4th Printing (1991) Monograph distributed by Calbiochem-Novabiochem (UK) Ltd

Sihra T.S., Nichols R.A., (1993) "Mechanisms in the regulation of neurotransmitter release from brain nerve terminals: current hypotheses" Neurochem. Res. **18**:47-58

Silva M.T., Guerra F.C., Magãlhaes M.M., (1968) "The fixative action of uranyl acetate in electron microscopy" Experientia **24**:1074

Simmons L.K., Schuetze S.M., Role L.W., (1990) "Substance P modulates single-channel properties of neuronal nicotinic acetylcholine receptors" Neuron **2**:393-403

Singer S.J., (1959) "Preparation of an electron-dense antibody conjugate" Nature (London) **183**:1523-1524

Singer S.J., Schick A.F., (1961) "The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin" J. Biophys. Biochem. Cytol. **9**:519-537

Sixma T.K., Pronk S.E., Kalk K.H., Wantna E.S., van Zanten B.A.M., Witholt B., Hol W.G.J., (1991) "Crystal structure of a cholera toxin-related heat-labile

enterotoxin from *E. coli*" Nature (London) **351**:371-377

Sjöstrand F.S.,(1953) "Ultrastructure of retinal rod synapses of guinea-pig eye" J. Appl. Phys. **24**:1422-1423

Slot J.W., Geuze H.J., (1985) "A new method of preparing gold probes for multiple-labelling cytochemistry" Eur. J. Cell Biol., **38**:87-93

Smith S.J., Augustine G.J., (1988) "Calcium ions, active zones and synaptic transmitter release" Annu. Rev. Neurosci. **10**:633-693

Sommer B.,Seeburg P.H.,(1992) "Glutamate receptor channels: novel properties and new clones" Trends Pharmacol. Sci. **13**:291-296

Somogyi P.,Takagi H.,Richards J.G.,Mohler H.,(1989) "Subcellular localization of Benzodiazepine/$GABA_A$ receptors in the cerebellum of rat, cat and monkey using monoclonal antibodies" J. Neurosci. **9**:2197-2209

Sossin W.S.,Fischer J.M.,Scheller R.R.,(1989) "Cellular and molecular biology of neuropeptide processing and packaging" Neuron **2**:1407-1417

Stein P.E., Boodhoo A., Tyrrell G.J., Brunton J.L., Read R.J., (1992) "Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*" Nature (London) **355**:748-750

Steinbach J.H., Zempel J., (1987) "What does phosphorylation do for the nicotinic acetylcholine receptor" Trends Neuro. Sci. **10**:61-64

Stevens C.F., (1993) "Quantal release of neurotransmitter and Long-Term Potentiation" Cell **72**/Neuron **10**:55-64

Stolerman I.P., Albuquerque E.X., Garcha H.S., (1992) "Behavioral effects of anatoxin, a potent nicotinic agonist in rats" Neuropharmacology **31**:311-314

Stollberg J., Berg D.K., (1987) "Neuronal acetylcholine receptors: fate of surface and internal pools in cell culture" J. Neurosci **7**:1809-1815

Suárez-Isla B.A., Hucho F., (1977) "Acetylcholine receptor: -SH group reactivity as indicator of conformational changes and functional states" FEBS Lett. **75**:65-69

Südhof T.C., Jahn R., (1991) "Protein of synaptic vesicles involved in exocytosis and membrane recycling" Neuron **6**:665-677

Sumikawa K., Houghton J., Smith J.G., Bell L., Richards B.M., Barnard E.A., (1982) "The molecular characterization and cloning of a cDNA coding for the alpha subunit of the acetylcholine receptor" Nucleic Acids Res. **10**:5809-5822

Swanson L.W., Simmons D.M., Whiting P.J., Lindstrom J., (1987) "Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system" J. Neurosci. **7**:3334-3342

Swope S.L.,Moss S.J.,Blackstone C.D.,Huganir R.L.,(1992) "Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity" FASEB J., **6**:2514-2523

Thorne B.,(1990) "Nicotinic regulation of acetylcholine release from the rat brain hippocampus" PhD thesis, Bath University

Thorne B., Wonnacott S., Dunkley P.R., (1991) "Isolation of hippocampal synaptosomes on Percoll gradients: cholinergic markers and ligand binding sites" J. Neurochem. **56**:479-484

Thureson-Klein A.K., (1983) "Exocytosis from large and small dense-cored vesicles in noradrenergic nerve terminals" Neurosci. **10**:245-252

Thureson-Klein A.K.,Klein R.L.,(1990) "Exocytosis from neuronal large-cored vesicles" Int. Rev. Cytol. **121**:67-126

Towbin H., Staehelin T.,Gordon J.,(1979) "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications" Proc Natl. Acad. Sci. USA **76**:4350-4354

Tzartos S., Lindstrom J.M., (1980) "Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits" Proc. Natl. Acad. Sci. **77**:755-759

Tzartos S.J., Rand D.E., Einarson B.E., Lindstrom J.L., (1981) "Mapping of surface structures of *Electrophorus* acetylcholine receptor using monoclonal antibodies" J. Biol. Chem. **256**:8635-8645

Umbriaco D., Watkins K.C., Descarries L., Cozzari C., Hartman B., (1991) "Non-junctional cholinergic (ACh) innervation of adult rat parietal cortex" 3rd IBRO World Congr. Neurosci. Montreal P54.2

Unwin N., (1993a) "Neurotransmitter action: opening of ligand-gated ion channels" Cell **72**/Neuron **10**:31-42

Unwin N., (1993b) "The nicotinic acetylcholine receptor at 9Å resolution" J. Mol. Biol. **229**:1101-1124

Valera S., Ballivet M., Bertrand D., (1992) "Progesterone modulates a neuronal nicotinic acetylcholine receptor" Proc. Natl. Acad. Sci. USA **89**:9949-9953

Van den Pol A.N., (1986) "Tyrosine hydroxylase immunoreactive neurons throughout the hypothalamus recieve glutamate decarboxylase immunoreactive synapses: a double pre-embedding immunocytochemical study with particulate silver and HRP" J. Neurosci **6**:877-891

Varndell I.M., Polak J.M., Eds (1984) "Immunolabelling for electron microscopy" Elsevier: Amsterdam

Vernallis, A.B.,Conroy W.G.,Berg D.K.,(1993) "Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes" Neuron **10**:451-464

Vernino S., Amador M., Luetje C.W., Patrick J., Dani J.A., (1992) "Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors" Neuron **8**:127-134

Villarroel A., Herlitze S., Loenen M., Sakmann B., (1991) "Location of a threonine residue in the α-subunit M2 transmembrane segment that determines the ion flow through the acetylcholine receptor channel" Proc. R. Soc. London (B) **243**:69-74

Villarroel A., Sakmann B., (1992) "Threonine in the selectivity filter of the acetylcholine receptor channel" Biophys. J. **62**:209-221

Vincent A., (1980) "Immunology of acetylcholine receptors in relation to Myasthenia Gravis" Physiol. Reviews **60**:756-824

Wada K., Ballivet M., Boulter J., Connolly J., Wada E., Deneris E.S., Swanson L.W., Heinemann S., Patrick J., (1988) "Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor" Science **240**:330-334

Wada E., McKinnon D., Heinemann S., Patrick J., Swanson L.W., (1990) "The

distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (α5) in the rat central nervous system" Brain Res. **526**:45-53

Wada E., Wada K., Boulter J., Deneris E., Heinemann S., Patrick J., Swanson L.W., (1989) "The distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system. A hybridization histochemical study in the rat" J. Comp. Neurol. **284**:314-335

Walker, J.F. (1964) "Formaldehyde" 3rd edn Reinhold: New York

Ward J.M., Cockcroft V.B., Lunt G.G., Smillie F.I., Wonnacott S., (1990) "Methyllcaconitine: a selective probe for neuronal α-bungarotoxin binding sites" FEBS Lett. **270**:45-48

Weakley, B.S. (1981) "A Beginner's Handbook in Biological Transmission Electron Microscopy" 2nd Edn Churchill: Livingstone

Whiting P., Esch F., Shimasaki S., Lindstrom J., (1987a) "Neuronal nicotinic acetylcholine receptor ß-subunit is coded for by the cDNA clone α4" FEBS Lett. **219**:459-463

Whiting P., Lindstrom J., (1986a) "Pharmacological properties of immunoisolated neuronal nicotinic receptors" J. Neurosci. **6**:3061-3069

Whiting P., Lindstrom J., (1986b) "Purification and characterization of a nicotinic acetylcholine receptor from chick brain" Biochemistry **25**:2082-2093

Whiting P., Lindstrom J., (1986c) "Purification and characterization of a nicotinic acetylcholine receptor from rat brain" Proc. Natl. Acad. Sci. USA **84**:595-599

Whiting P., Lindstrom J., (1987) "Purification and characterization of a nicotinic acetylcholine receptor from rat brain" Proc. Natl. Acad. Sci. USA **84**:595-599

Whiting P.J., Lindstrom J., (1988) "Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies" J. Neurosci. **8**:3395-3404

Whiting P.J., Liu R.S., Morley B.J., Lindstrom J., (1987b) "Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterized using monoclonal antibodies" J. Neurosci. **7**:4005-4016

Whiting P.J., Schopfer R., Swanson L.W., Simmons D.M., Lindstrom J.M., (1987c) "Functional acetylcholine receptor in PC12 cells reacts with a monoclonal antibody to brain nicotinic receptors" Nature (London) **327**:515-518

Whittaker V.P. (1969) "The synaptosome" in "Handbook of Neurochemistry" Lajtha A., (ed), Vol 2, Plenum Press: New York, pp327-364

Williams, M.A. (1977a) "Autoradiography and immunocytochemistry", Vol. 6, Part

I of "Practical Methods in electron microscopy" Ed Glauert, A.M., 1st edn, Elsevier: North-Holland Biomedical Press:Amsterdam.

Williams, M.A. (1977b) "Quantitative methods in biology", Vol. 6, Part II of "Practical Methods in electron microscopy" Ed Glauert, A.M., 1st edn, Elsevier, North-Holland Biomedical Press: Amsterdam.

Wisden W., Seeburg P.H., (1992) "$GABA_A$ receptor channels: from subunits to functional entities" Curr. Opin. Neurobiol., **2**:263-269

Wonnacott S., (1987a) "Editorial: Brain nicotinic binding sites" Hum. Toxicol. **6**:343-353

Wonnacott S.,(1987b) "Neurotoxin probes for neuronal nicotinic receptors" from "Neurotoxins and their pharmacological implications" ed Jenner P.,Raven Press: New York pp 209-231

Wonnacott S., (1990) 'The paradox of nicotinic acetylcholine receptor upregulation by nicotine" Trends Pharmacol. Sci. **11**:216-219

Wonnacott S.,Drasdo A.,Sanderson E.,Rowell P.,(1990a) "Presynaptic nicotinic receptors and the modulation of transmission release" in The biology of nicotine dependence. Ciba Foundation Symposium 152. eds Bock G., Marsh J., Wiley: Chichester pp87-101

Wonnacott S., Harrison R., Lunt G.G., (1982) "Immunological crossreactivity between the α-bungarotoxin binding component from rat brain and nicotinic acetylcholine receptor" J. Neuroimmunol. **3**:1-13

Wonnacott S., Irons J., Rapier C., Thorne B., Lunt G.G., (1989) "Presynaptic modulation of transmitter release by nicotinic receptors" Prog. Brain Res. **79**:157-163

Wonnacott S.,Jackman S.,Swanson K.L.,Rapoport H.,Albuquerque E.X.,(1992) "Nicotinic pharmacology of anatoxin analogues: II side chain structure-activity relationships at neuronal nicotinic ligand binding sites" J. Pharmacol. Exp. Ther. **259**:387-391

Wonnacott S., Russel M.A.H., Stolerman I.P., (1990b) editors of "Nicotine Psycopharmacology: molecular, cellular and behavioural aspects" Oxford University Press: New York

Wonnacott S.,Wilkie G.,(1991) "Enrichment of presynaptic cholinergic receptor binding sites on Percoll gradients" Abs. 21st Annu. Soc. Neurosci. **17**:384.17

Wray B.E., Sealock R., (1984) "Ultrastructural immunocytochemistry of particulate fractions using polyvinyl-chloride microculture wells" J. Histochem. Cytochem. **32**:1117-1120

Yamamura H.I.,Enna S.J.,Kuhar M.J.,(Eds) (1985) "Neurotransmitter receptor

binding" 2nd edn Raven Press: New York

Yoshida K., Engel J., Liljequist S., (1982) "The effect of chronic ethanol administration on high affinity ^{3}H-nicotine binding in rat brain" Arch. Pharmacol. **321**:74-76

Yoshida K., Imura M., (1979) "Nicotinic cholinergic receptors in brain synaptosomes" Brain Res. **172**:453-459

Zucker R.S.,(1989) "Short-term synaptic plasticity" Annu. Rev. Neurosci. **12**:13-31

Appendix A The choices available for immunoelectron microscopy of synaptosomes

A.1 Conditions for similar immunoelectron microscopy studies

The optimal conditions for the examination of synaptosomes by electron microscopy were established in the late 1960's (review: Jones, 1975). Briefly, synaptosome suspensions were fixed by dual fixation (glutaraldehyde, then osmium tetroxide, as Sabatini *et al*, 1963), dehydrated with acetone, embedded in expoxyresin (Glauert and Glauert, 1958) and positively stained using uranyl acetate (Silva *et al*, 1968) and lead citrate (Reynolds, 1963) salts.

Immunoelectron microscopy experiments, using neuronal tissue blocks, tend to follow a very similar pattern (eg: Barnes *et al*, 1988, 1992; Hill *et al*, 1993; Dechesne *et al*, 1990; Peng *et al*, 1991; Sargent *et al*, 1989; Somogyi *et al*, 1989) with only minor variations. Briefly, whole brains were fixed by perfusion and then dissected (Barnes *et al*, 1988), or sometimes dissected regions were fixed by submersion (Sargent *et al*, 1989). This primary fixation was aldehyde based with a phosphate buffer vehicle, the fixative(s) were either paraformaldehyde alone (Peng *et al*, 1991), 4% paraformaldehyde/0.1% glutaraldehyde (Barnes *et al*, 1988, 1992; Dechesne *et al*, 1990; Matsas *et al*, 1986), paraformaldehyde/acrolein (Sargent *et al*, 1989), paraformaldehyde/picric acid (Hill *et al*, 1993) or paraformaldehyde/glutaraldehyde/picric acid (Somogyi *et al*, 1989). The tissue

was then cryoprotected, with sucrose in phosphate buffer, frozen and sectioned (50μm). Mounted or "free floating" sections were immunostained, using a phosphate buffer vehicle, then fixed with osmium tetroxide (1% v/v). Sections were dehydrated, embedded, sectioned on an ultramicrotome (100nm) and positively stained essentially as described above.

One approach to immunostaining subcellular particles was described by Wray and Sealock (1984), La Rochelle *et al* (1985) and Ratman *et al*, (1986a): they were immobilized onto poly(vinyl chloride), lysed and immunostained before fixation (glutaraldehyde/tannic acid), dehydration (ethanol) and embedding (Epon). An alternative approach to immunostaining subcellular particles was to immunostain them in free suspension (Lentz and Chester, 1977) and then pelleted before fixation (glutaraldehyde), dehydration (ethanol) and embedding (Epon).

The following sections describe the available choices of preparative conditions and the reasons for using the two representative approaches described in section 4.

A.2 Immunostaining techniques

Immunostaining may be performed at virtually any stage in specimen treatment, but some stages tend to be more appropriate. As antibodies are often raised against native receptors an obvious stage to immunostain a specimen is before any chemical treatment whatsoever, this avoids epitope denaturation problems and so conditions of fixation, dehydration, embedding and positive staining can be chosen for optimal image quality. Despite this advantage **prefixation immunostaining**

(Hayat, 1989a) is rarely used, because it produces optimal results only when epitopes are accessible without any pretreatment and very few epitopes fall into this category. Membrane permeabilization or lysis can be sometimes employed to allow immunological access (Ratnam, 1986a), but this approach can be unacceptable if a morphological identification is required which involves complicated membraneous structures, and also it causes antigen migration (Beesley, 1989). Fortunately, part of the nAChR is extracellular and some Lindstrom antibodies are directed against epitopes which are at that extracellular location (Ratnam *et al*, 1986b).

Cryoscopic preservation techniques (Hayat, 1989a) were inappropriate prior to prefixation immunostaining because:

a) the relevant equipment (and expertise) was not available

b) cryoscopic sections of synaptosomes can disperse, as they melt, during antigen exposure, or the synaptosome density can be high enough to prevent colloidal gold penetration.

Thus unfixed synaptosomes could have been immunostained in free suspension (Lentz and Chester, 1977) or immobilized against a matrix (Ratnam *et al*, 1986a). A potential advantage of immunostaining immobilized synaptosomes is the reduced probability of immunogold dissociation (Hayat, 1989b), but one dimension is lost.

Post-embedding immunostaining is performed immediately after sectioning. In most cases it is pointless to immunostain a specimen at any time between prefixation and post-embedding stages, because epitope access is constant until

sectioning, but the chance of recognition falls with increasing denaturation during specimen treatment. After sectioning, access does not increase further, but positive stains alter biomolecule conformation. Post-embedding immunostaining is a common approach (Hayat, 1989a), it offers:-

a) equal access to all epitopes

b) virtually any immunostaining conditions can be used

c) no problems of dissociation

d) minimal damage to tissue morphology

This technique does have disadvantages:-

a) antigen denaturation is almost inevitable and so there is high chance of recognition failure

b) methods to increase the chance of recognition involve a decrease in image quality and clarity

c) probe penetration into resin is poor (Beesley, 1989) so that, at best, only 10% of epitopes are available for recognition

The choice of immunostaining techniques. Both prefixation and postembedding techniques were evaluated to determine the viability of immunostaining the $\alpha 4\beta 2$ receptor in subcellular fractions. The receptor is present at a low concentrations in subcellular fractions and the probability of detection was small, so prefixation immunostaining was performed on synaptosomes in free suspension, to optimise access and to maximise the number of synaptosomes/field. The possibility of probe dissociation was examined by performing controls (section 3). If immunolocalisation and immunostaining proved viable, then immunopurified

synaptosomes would probably be best examined by matrix digestion (Richardson *et al*, 1984), synaptosome immobilization (Ratnam, 1986a), then prefixation immunogold staining.

A.3 The immunostain

Immunostaining is a natural extension of conventional chemical positive staining (A.11), combining the specificity of biomolecules with the electron dense characteristics required for successful staining. Early immunocytochemistry employed ferritin coupled antibodies (Singer, 1959, Singer and Schick, 1961), later other markers including enzyme conjugated- (Deutch *et al*, 1987; Hill *et al*, 1993) and radiolabelled- (Swanson *et al*, 1987) antibody probes have been used. Colloidal particles have distinct profiles and offer superior localization and quantification to the radiolabelled and enzyme-linked probes (Williams, 1977b), but they suffer from reduced access (Williams, 1977a) and increased dissociation (Hayat, 1989b). Colloidal particles had to be used for this work, because the other two probe types could not localise an antigen to one side of the synapse (Hill *et al*, 1993).

Colloidal gold is classically considered the best colloidal particle for immunostaining (reviews: Beesley, 1989; Hayat, 1989a, b; Horisberger, 1981; Horisberger and Rosset, 1977; Polak and Van Noorden, 1984; Weakley, 1981). Gold labelling, initially using gold-tagged IgG (Faulk and Taylor, 1971), resolves the combination of problems found with the other probes:-

1) Colloidal gold particles are spherical, electron dense particles, which are readily detectable in electron micrographs.

2) Nonspecific staining of the resin by the gold particles is extremely low.

3) The particles are discrete and bind with high affinity, allowing direct quantification.

4) Gold particles can be made from the reduction of gold chloride (Horisberger and Rosset, 1977) in a range of sizes (2.6nm to 150nm are available although 5 to 20nm are generally used) by the manipulation of the preparative conditions (eg Horisberger, 1981; Slot and Geuze, 1985). The control of particle size, and the close proximity of the particle and recognition site means that localization is excellent.

5) Gold particles can be attached, via complex electrochemical interactions, to a variety of biomolecular probes, eg lectins, hormones, immunoglobulins, glycoproteins, lipoproteins, toxins, dextran, Avidin, Protein A or G and enzymes. The non-covalent attachment is generally not conformationally disruptive and so the probe retains its biological activity and specificity.

6) Multiple labelling can be routinely performed, using gold particles of two or more sizes.

7) Gold particles can be used as seeds for silver ion deposition (Holgate *et al*, 1983; Hayat, 1989b; James, 1977) this improves gold particle visibility and silver staining is visible by light microscopy allowing comparative studies.

8) The technique is flexible:- pre- and post-embedding labelling may be performed, the latter is more common, and thin conventional sections, as well as thin cryosections, can be stained (as A.1).

Gold conjugated probes do have some disadvantages (Beesley, 1989; Hayat,

1989a, b; Williams, 1977a):-

1) The particles are dense and can easily dissociate during centrifugation.

2) The particles are relatively large and so cannot penetrate resin.

3) The labelling efficiency is proportional to the inverse of the square root of particle diameter, but visibility decreases with particle size, this can be compensated by silver enlargement.

Initial studies were performed using 10nm diameter gold particles in order to determine an appropriate particle size and to avoid artifacts from silver enhancement. Once a successful immunostaining procedure had been derived, the particle size would be reduced to a minimum for immunostaining and silver enhancement used for visualisation. Avidin-gold probes (Hill *et al*, 1993) were used for prefixation immunostaining after specificity problems were encountered during postembedding experiments, the Avidin-biotin system combines high specificity with high affinity (Polak and Noorden, 1984) and so reduces the probability of dissociation.

A.4 The synaptosome preparation

The concentration of nAChRs vary throughout the brain (Clarke *et al*, 1985) and the neuronal distribution varies in different brain regions (sections 1.4,3.4.3),but estimates of the size of the presynaptic subpopulation for any particular region vary considerably. Synaptosomes were prepared from whole brains (each minus cerebellum), rather than homogenized brain regions, despite the reduced $\alpha 4\beta 2$ concentration, in order to avoid choosing a region with an untypical distribution or concentration of nAChRs. If brain region synaptosomes had been examined,

the cortex would have been examined first (sections 1.4, 3.4.3).

The choice of synaptosome preparation had to be made empirically, three obvious options were available:-

a) The S1-Percoll method (section 3.2.4). A high proportion of the structures in fraction F4 are synaptosomes (Dunkley *et al*, 1988) and preliminary evidence indicates the F4 fraction contain high concentrations of the α4ß2 (Barnes *et al*, 1992; Thorne *et al*, 1991).

b) The P2-sucrose gradient method (section 3.2.2). The P2b fraction contains a fairly high proportion of synaptosomes (Whittaker, 1969) and high concentrations of the α4ß2 (Yoshida and Imura, 1979).

c) The H-sucrose gradient method (section 3.2.2). The Hb fraction contains a moderately high proportion of synaptosomes (Rapier *et al*, 1988) and high concentrations of the α4ß2 (Rapier *et al*, 1990).

Options (a) and (c) do not involve synaptosomal precipitation (by centrifugation) and resuspension, which cause synaptosomal damage (Dunkley *et al*, 1988), but (a) does produce a low yield of synaptosomes (Dunkley *et al*, 1987) containing high concentrations of Percoll. This dense medium can be removed by centrifugal washing (Thorne, 1990; section 3.3.3), but this causes synaptosomal damage. Prefixation immunostaining involves centrifugal washing which must be performed at higher, more destructive, speeds if the Percoll is still present. Percoll associates with synaptosomal membranes (Wonnacott, person. comm.), and its effects upon post-embedding immunostaining are not known, and so removal is desirable. The efficiency of the Percoll preparative method raises 2 problems:-

i) the high synaptosomal content of Percoll fractions (F4) means there are few other structures to act as controls to allow the interpretation of immunogold staining.

ii) synaptosomes are spread over all Percoll fractions, the morphology and neurotransmitter content of synaptosomes vary with each fraction so that each fraction, is unrepresentative of the entire synaptosome population (Harrison *et al*, 1988), this is a more serious problem for an assessment of nAChR distribution.

The use of technique (a) was rejected, until immunostaining could be established. Experiments were performed to compare the synaptosome content and quality of the Hb and the P2b preparations (section 4.3.1.1).

A.5 Synaptosome damage

Synaptosomes are delicate structures and can be more easily damaged than synapses in a tissue block. Synaptosomal damage can be caused by:-

a) **Mechanical disruption** when a synaptosome suspension is washed by centrifugation and resuspension (Dunkley *et al*, 1988; Fleming *et al*, 1980). Mechanical damage is a major consideration because of the extensive centrifugal washing during prefixation immunostaining described in section 4.2: postembedding immunostaining methods involve very little centrifugal washing before embedding, so mechanical damage was less of a problem.

b) The use of detergents, eg **Triton**, during immunostaining. Triton has no effect upon synaptosome damage during **post-embedding immunostaining**, as the synaptosomes have been fixed and embedded (section 4.3.4). The use of Triton for **pre-fixation immunostaining** is impractical, because synaptosomes are lysed

and the damage to these delicate membraneous structures renders them unidentifiable.

c) The use of vehicles of inappropriate osmolarity, pH or chemical content (Hayat, 1989a, Weakley, 1981) can cause tissue damage:-

i) Tissue damage can be caused by the **pH** of vehicles, synaptosome disruption is least likely when buffers were made with physiological pH (7.4, Hayat, 1989a).

ii) Isotonic, or slightly hypertonic, vehicles are generally used to minimize tissue disruption, however the actual optimal osmotic strength of vehicle varies considerably depending on the specimen tissue (Hayat, 1989a) and so osmotic strength must be empirically defined. In this specific case, the strength of synaptosome membranes is low and so isotonic buffers are likely to be iso-osmotic with synaptosome content (320-350 mosmol: Dunkley *et al*, 1987). The osmotic strength of fixatives is quite high, but they penetrate structures slowly, an empirical rule (Hayat, 1989a) is to use vehicles isotonic to tissues.

iii) The chemical content of buffers can cause or reduce synaptosomal disruption. Some chemicals tend to help preserve tissue structure, eg sucrose (Burdett and Rogers, 1970) and calcium chloride (Morel *et al*, 1971). Calcium chloride was avoided, because it reacts with several chemicals to give insoluble electron dense precipitates (Hayat, 1989a). The use of dense media (eg 0.32M sucrose) tends increase mechanical disruption during centrifugation as high forces must be used (Dunkley *et al*, 1988).

A.6 Vehicle choice

Vehicle usage affects immunostaining and synaptosome integrity, but it can also affect image quality, and chemical cross-reaction can cause the precipitation of

electron dense particles during staining (A.11). Several buffers are routinely used, they have differing effects on fixation and extraction. The commonly used buffers are based on the following salts: cacodylate, collidine, HEPES, MOPES (3-[N-morpholino] propanesulphonic acid), phosphate, PIPES (piperazine-N,N'-bis[2-ethanesulphonic acid]), Tris and veronal acetate. A variety of chemicals are added to stabilize tissue, such as sucrose and calcium chloride, or to alter osmolarity, such as sodium chloride. A full discussion of the relative merits of vehicle components is given elsewhere (Hayat, 1989a; Lewis and Knight, 1977), the relevant points are detailed, as appropriate, in other sections. It is generally recommended that optimal buffer conditions are established empirically (section 4.3.1.3).

A.7 Fixation method

There are four methods of chemical fixation:

a) vascular perfusion (Barnes *et al*, 1988)

b) immersion (Sargent *et al*, 1989)

c) dripping onto the surface of the organ (Hayat, 1989a)

d) injection into the organ (Koelle *et al*, 1974)

Method (a) is the best option and method (b) the worst option for most cases, because of the difficulty of access (Hayat, 1989a). In this project, the specimen is a suspension of small particles and the situation is reversed, because the distance the fixative must penetrate is so short (Reith *et al*, 1984). The immersion method of fixation was used throughout these experiments and care was taken to minimize the penetration distance.

A.8 Fixative choice

Different fixatives were chosen for prefixation and postembedding immunostaining experiments.

A.8.1 Prefixation immunostaining

Pre-fixation immunostaining is unaffected by receptor denaturation, thus the criteria for fixation must be image quality. The dual fixation technique (Sabatini *et al*, 1963) consists of primary fixation with glutaraldehyde then secondary fixation using osmium tetroxide. Glutaraldehyde is the most effective aldehyde for preserving fine structure (Sabatini *et al* 1963,). Fixation chemistry is not completely understood because commercial glutaraldehyde is highly impure and mainly consists of polymerised condensation derivatives (reviews Hayat 1986a, 1989a; Sabatini *et al* 1963). Glutaraldehyde reacts mainly with proteins, some secondary structure (especially α helical arrangements) is lost during cross-linkage, but tertiary or quaternary structure are usually unaffected, thus denaturation is limited and often biological function and conformation are maintained.

Extraction, during osmium tetroxide, is reduced by the glutaraldehyde fixation. Osmium tetroxide acts as a fixative and an electron dense positive stain. It is a reactive chemical which primarily cross-links the C=C double bonds of lipids and lipoproteins (Dreher *et al*, 1967), but also reacts with proteins, polypeptides (Hake, 1965), nucleic acids (Chang *et al*, 1977) and a variety of other compounds including phenols and alkaloids (review Hayat, 1989a).

A.8.2 Post-embedding immunostaining

Osmium tetroxide causes considerable conformational distortion and chemical modification of proteins (Lenard and Singer, 1968; Hayat, 1989a), this denaturation often results in the loss of antigenic sites. It can sometimes be used prior to immunostaining (Bendayan and Zollinger, 1982), but generally this gives poor or negative results (Beesley, 1989, Polak and Van Noorden, 1984). Permanganates cause extensive extraction, which results in clear membranes by a dramatic reduction of non-lipid components (Hayat, 1989a). As the concentration of nAChRs is low anyway, increased extraction was inappropriate.

Aldehydes are effective for the maintenance of fine structure of proteins and proteinaceous structures (Hayat, 1989a). Fixation is by condensation reactions between the aldehyde and amino groups yield alpha-hydroxyamines, which then cross link with other amino groups by further condensation reactions. The first aldehyde to be used was the monoaldehyde acrolein. In the early 1960's Barrnett and colleagues (Sabatini *et al*, 1963) published extensive studies with aldehydes and began their general usage. Acrolein cannot be used with sucrose (Hayat, 1989a) and so it was avoided. Glutaraldehyde is described in section A.8.1. Formaldehyde (methanal) is a far weaker and less specific cross-linking agent than glutaraldehyde. The colourless gas readily dissolves in water to give formalin, which contains contaminants, such as methanol and methanoic acid (Walker, 1964), that inhibit cross-linking, so formaldehyde solutions are best prepared from the polymer paraformaldehyde, by hydrolysis, under mild alkaline conditions. Formaldehyde reversibly cross-links proteins and nucleic acids, and seems to encourage the extraction of lipids during subsequent dehydration.

The mixed aldehyde fixation technique minimizes denaturation, whilst maximizing tissue preservation, and so it is often used prior to immunostaining (Barnes *et al*, 1988, 1992; Dechesne *et al*, 1990; Matsas *et al*, 1986). Formaldehyde and glutaraldehyde (Karnovsky, 1965) seem to act in concert, to give a far greater fixation than would be expected from the individual aldehydes. The classical rationale for this effect is that the former rapidly penetrates the material, causing partial fixation until the slower glutaraldehyde arrives, to cause irreversible cross-linkage, but it seems increasingly likely that the two aldehyde react with each other to catalyze cross-linkage (Kirkeby and Moe, 1986).

Other fixatives (Hayat, 1981, 1986b), including tannic acid (Chaplin, 1985) and picric acid (Somogyi *et al*, 1989), can be used in combination with aldehyde fixation to improve membrane visualization, but for initial experiments it was important to minimise the use of potentially denaturating chemicals.

A.9 Fixation conditions

In order to minimise artifacts, the following fixation conditions have been established empirically (Hayat, 1989a):-temperature, duration, and method of fixation, and buffer composition, concentration, pH and osmolarity. A major factor to consider was the degree biomolecule extraction (leeching). Fixation reduces extraction, but the fixatives themselves cause extraction.

The rate of extraction during **glutaraldehyde** exposure is very low, it is increased at higher temperatures, longer durations and high fixative concentrations, but there is a wide window for these parameters and penetration is affected by the

same variables. Minimal artifacts are generally achieved by using 1-4% glutaraldehyde (optimum 2%), for 1-18 h (optimum 2h) at 0°C to room temperature (optimum 4°C). The pH for fixation is optimal at 7.0-7.4:-as pH increases cross-linking improves, but at pH's greater than 7.5 the aldehyde tends to polymerize, also a physiological pH tends to minimize artifacts from tissue damage.

Osmium tetroxide fixation causes extensive extraction, especially of unfixed material. The molecule is large and non-polar, it penetrates slowly (0.7mm per hour), but is unimpeded by layers of charged material. Minimal artifacts are generally achieved by using 0.5% and 2% osmium tetroxide (optimum 1%), for 30-120 minutes (optimum 30-45 min for particular material) and physiological pH's (7.2-7.4). Fixation is best begun at 4°C, although the temperature can then be allowed to rise to room temperature.

A.10 The resin and dehydration conditions

Electron beams are rapidly attenuated, furthermore stereological information deteriorates with section thickness (Holmes Effect, 1921, see Williams, 1977b), thus image quality and clarity increase with decreasing section thickness providing sufficient contrast can be achieved by staining. Section thickness can be reduced by using a sharper knife and mechanical cutting (A.11), and by making the specimen harder and more rigid. Embedding is defined as the complete and uniform impregnation of the interstices of a specimen with a medium and its subsequent solidification, resulting in a large and uniform increase in specimen rigidity. This is often achieved by monomer infiltration, ie the gradual and

continuous replacement the original solvent (water), then polymerization. Infiltration is accelerated by limiting specimen size and by continuous agitation. Various embedding materials are available (Hayat, 1989a).

Epoxyresin was a suitable resin for prefixation immunostaining where denaturation is unimportant, but image quality was desired. Epoxyresin (araldite) is a glycerol-based aromatic resin (Glauert and Glauert, 1958; Hayat, 1989a), which is relatively hard and stable to electron beams and so sections have negligible cutting artifacts and can be mounted directly on grids, therefore specimens can be viewed with good image quality and negligible artifacts to a high resolution. The disadvantages of araldite are mainly related to its hydophobicity and immiscibility with water:-

1) All free water must be replaced by a resin-miscible solvent before resin infiltration. The best solvent for this extensive dehydration in terms of: resin miscibility, minimization of structural changes, rapidity of dehydration and hence minimal extraction, and finally low chemical reactivity is acetone. A **series of acetone washes** result in gradual, even and **complete dehydration**. The hydrophobic conditions contribute to protein denaturation and loss of antigenicity

2) The fixation must be more severe to minimize the extraction during full dehydration, this increases protein denaturation and loss of antigenicity.

3) Polymerization is achieved using a "heat cure" procedure whereby the resin-impregnated specimen is heated to 60°C, for 48 hours, this increases protein denaturation and loss of antigenicity.

4) The density of the resin and hydrophobic character of the resin does not favour immunological staining. This problem may be bypassed by etching sections, but

this process of breaking bonds risks specimen damage and also results in loss of the positive resin characteristics of strength and stability so, for example, electron beam damage is more likely. Etching is pointless if the antigen has been denatured to a degree where epitope recognition is impossible.

Epoxyresin was of doubtful value for post-embedding immunostaining because of protein denaturation. Previous work, using Lindstrom monoclonal antibodies (Thorne, 1990, personal comm.), failed to show specific post-embedding immunogold staining of $\alpha 4\beta 2$ nAChRs in epoxyresin embedded P2b preparations. A far more appropriate resin was LR White (Newman *et al*, 1983, 1989; Hayat, 1989a), which is a polar resin, because the monomer is a polyhydroxylated aromatic acrylic. LR White is miscible with water (if $H_2O < 12\%$ v/v), so dehydration can be rapid, incomplete and use the milder solvent ethanol. Infiltration is rapid because of the acrylic resin's low viscosity. Thus, extraction and denaturation are minimized during dehydration and embedding. Immunocytochemicals can penetrate sections without etching as the resin is hydrophillic and has a relatively open structure (Newman, 1987). The resin has very low toxicity and during immunostaining the resin itself is negligibly stained. LR White polymerization requires the absence of oxygen, but can be achieved in three ways:-

a) Heat curing. Full (60°C) or partial (55°C) curing takes about 24 hours, so denaturation is high, but less than attained when curing epoxyresin.

b) Chemical curing. Chemical catalysts and modifiers can be used to polymerize the resin, but they cause denaturation both chemically and by virtue of high local temperatures from highly exothermic polymerization reactions.

c) Light curing. The use of UV radiation to cure LR White is by far the least damaging curing procedure.

The resin could not be cured using UV-light for practical reasons, but the other two curing methods were assessed. The disadvantages of LR White are related to its low density:- it is soft and difficult to cut so images are less clear; beam stability is poor, so sections tend to need to be mounted on coated grids; both the lower resin density and use of coated grids decrease image quality.

A.11 Sectioning and positive staining

Sections were produced, at a range of section thickness, using an ultramicrotome with both mechanical and thermal feed. Two types of knife were available (review: Hayat, 1989a):

a) Diamond knives are very sharp and cut very clear sections down to a thickness of 5-10nm, they are very wear resistant, but they are expensive and only moderately tough.

b) Glass knives are cheap and disposable, but they are not wear resistant and are less sharp, they produce inferior images. Glass knives were considered adequate and for financial reasons they were used for this project.

When sections have insufficient strength, either to withstand the electron beam or to self support when mounted directly onto grids, a support film is required. The film must have high electron transparency to reduce artifacts, whilst providing strength and electron stability. As artifacts are always introduced, support films are only used when necessary. Preliminary experiments (section 4.3.3) showed that LR White resin had insufficient electron stability. LR White sections were

mounted on carbon coated piloform support films. Piloform is a good physical support, but has little electron stability, the carbon coating provides beam stability (Hayat, 1989a).

All sections were positively stained, after all other treatments, by standard techniques (Reid, 1974), using standard stains (Lewis and Knight, 1977): lead citrate (Reynold, 1963) and uranyl acetate (Weakley, 1981). When osmium tetroxide fixation was performed it also acted as a positive stain (Hayat, 1989a). A positive stain improves image contrast for the structure of interest by making some biomolecules more electron dense. Image opacity increases with electron density, caused by the degree of electron scatter, which is determined by atomic weight, section thickness and density, and by microscope parameters such as accelerating voltage and aperture size. Staining helps image contrast and resolution, facilitates focusing and also acts to stabilize stained components so that they are less prone to sublimation by the electron beam, this latter factor leads to even greater contrast. Staining tends to be additive and so both stains were employed. Chemical cross-reactions between positive stain and any vehicles is possible (Hayat, 1989a; Lewis and Knight, 1977), but this is only a consideration when washing is inadequate (section 4.3.1,4.3.3).

Appendix B A screening protocol to find antibodies for the immunoadsorption and immuno-electron microscopy of synaptosomes

Future antibodies should be screened to verify receptor recognition before attempting immunoadsorption and immunoelectron microscopy experiments. The following procedure is suggested:-

1) If the antibody is raised in the same species as the preparation bearing the nAChR, then the antibody should be biotinylated to avoid specificity problems.

2) Confirm the secondary probe will recognize the antibody, first by dot-blot recognition (section 3.3.5.2), then by using the secondary probe to attach the antibody to the bead (section 5.3.1)

3) Confirm that the antibody can be used to immunoprecipitate detergent solubilized receptor (section 5.3.2)

4) Confirm that the antibody can be used to immunostain the receptor in the absence of Triton (section 3.3.7) using an ^{125}I-labelled secondary probe.

Any further problems in immunoadsorption or immunoelectron microscopy probably reflect probe dissociation or low receptor concentrations.

Appendix C Plates

Summary

Plate 1a Micrograph of Hb demonstrating analysis field

Plates 1b and 1a show a representative field at the magnification used for analysis (30k) and the total analysis field at a slightly lower magnification respectively. The scale bar in 1a shows 1,000nm. For specimen and treatment details see plate 1b legend.

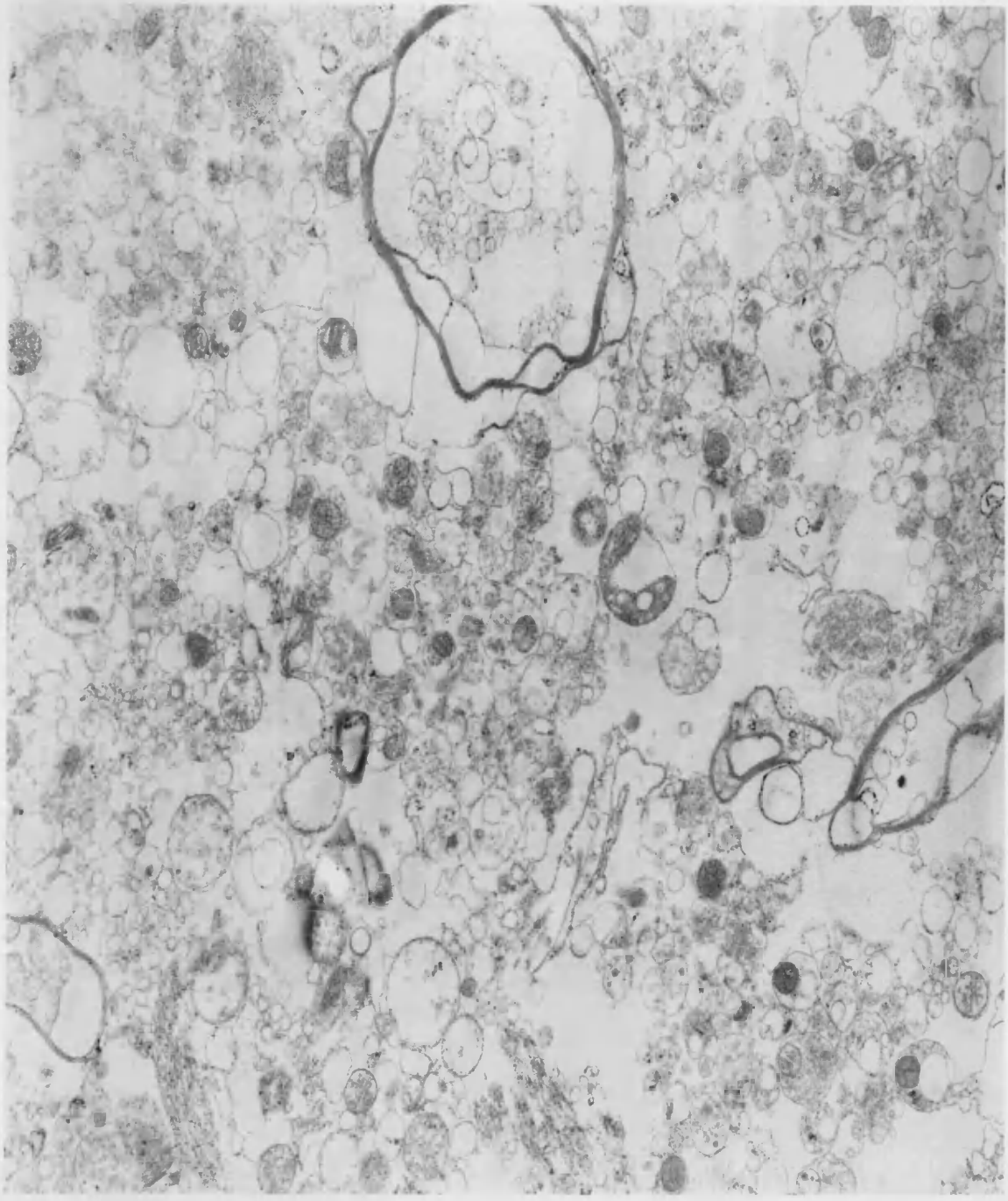


Plate 1b

Plates 1b shows a representative field at the magnification used for analysis (30k) of plate 1a.

Specimen:- Hb. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS. Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none. Fixation:- Glutaraldehyde (o/n, 4°C), then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS. Encapsulation:- None. Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/ lead citrate

Results:-

Synaptosomes (s) were visible as plasma membrane (pm) enclosed structures of approximately 0.5 μm diameter, containing synaptic junctions (j), mitochondria (m) and synaptic vesicles (sv). Background staining (b) was visible. A variety of non-synaptosomal structures were also seen in micrographs of Hb preparations.

Plate 2

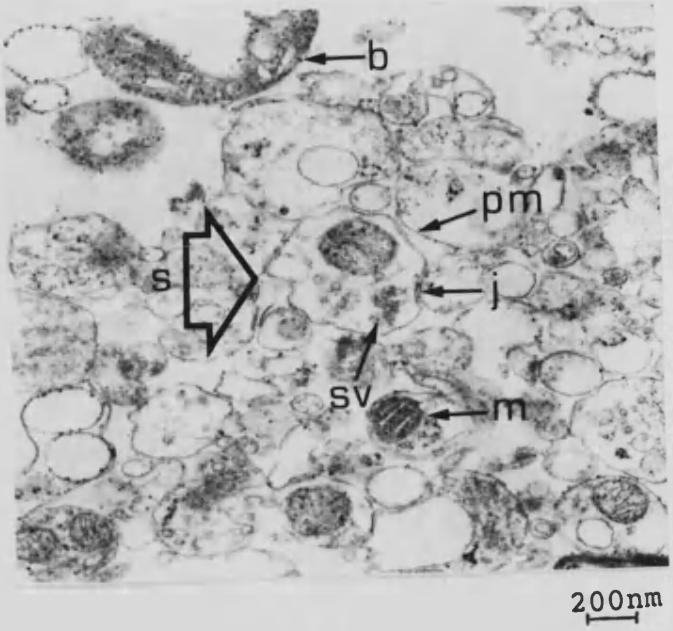
Plates 2 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS. Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none. Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS. Encapsulation:- None. Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

Synaptosomal damage is greater than in Hb (plate 1), but the number of identifiable synaptosomes is greater and the number of non-synaptosomal structures is far lower in the P2b preparation (plate 2).

1b



2

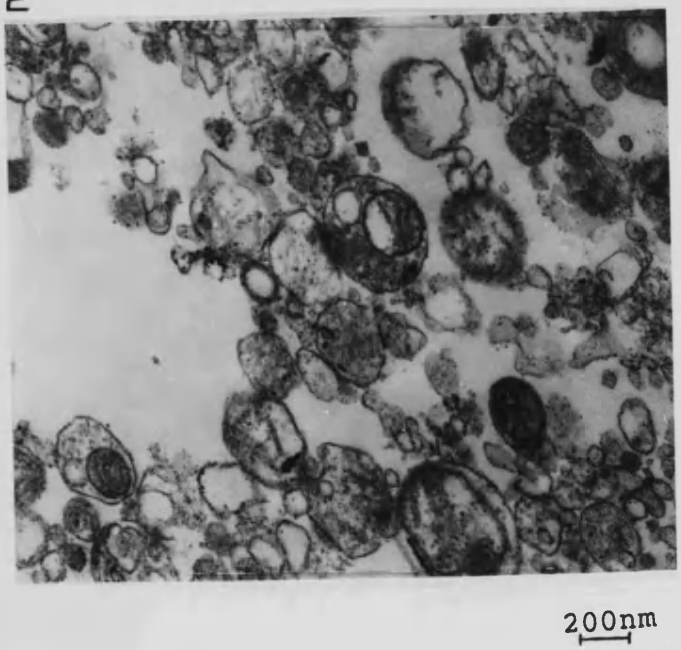


Plate 3

Plates 3 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- prefixation. Immunostaining vehicle:- PBS

Concentration of mAb:- 270 (200 pM), 290 (3375 pM), 297 (38 pM), 299 (38 pM) in 1ml

Secondary probe:- Avidin-10nm gold (1.5×10^{12} particles, 1ml). Blocking (immunostain):- none

Fixation:- Glutaraldehyde (1h, 4°C) then osmium tetroxide (1h, 4°C).

Fixation vehicle:- PBS then sucrose for OsO_4. Encapsulation:- Pellet after osmium fixation

Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf

Positive stains: - Uranyl acetate/ lead citrate

Results:-

Synaptosomal damage is greater than in P2b (plate 2), reflecting greater centrifugation. Gold staining (g) could be distinguished from background staining (b) with care.

Plate 4

Plates 4 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

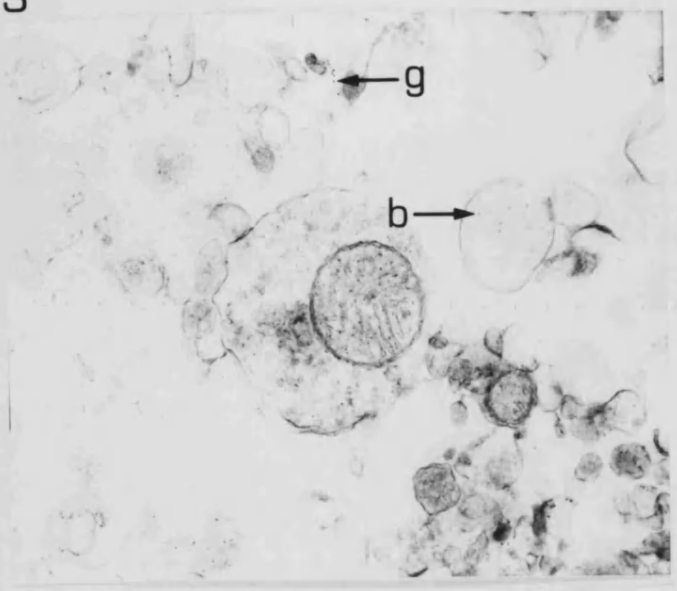
Encapsulation:- Agar. Dehydration:- Acetone. Embedding medium:- Epoxyresin.

Mould:- Eppendorf. Positive stains:- Uranyl acetate/ lead citrate.

Results:-

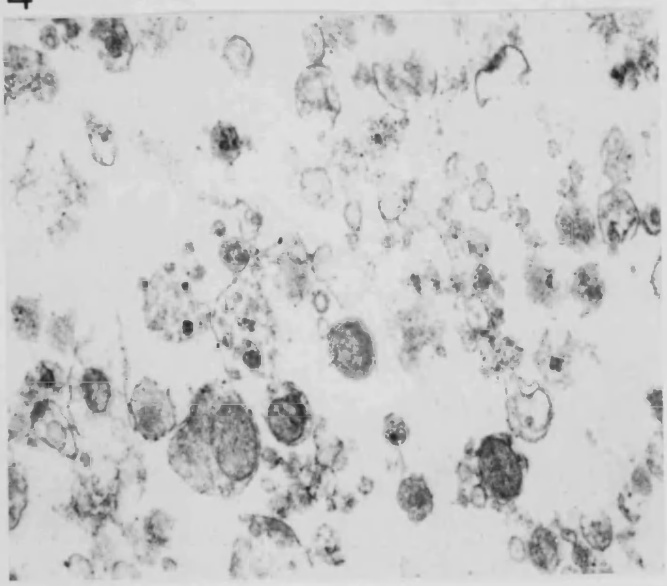
All synaptosomes were damaged, this was thought to be due to the high temperature (50°C) of the agar.

3



200nm

4



200nm

Plate 5

Plates 5 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS. Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none. Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- 100mM phosphate (pH 7.4). Encapsulation:- Agar. Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/ lead citrate

Results:-

All synaptosomes were damaged, this was thought to be due to the high temperature (50°C) of the agar (see plate 4).

Plate 6

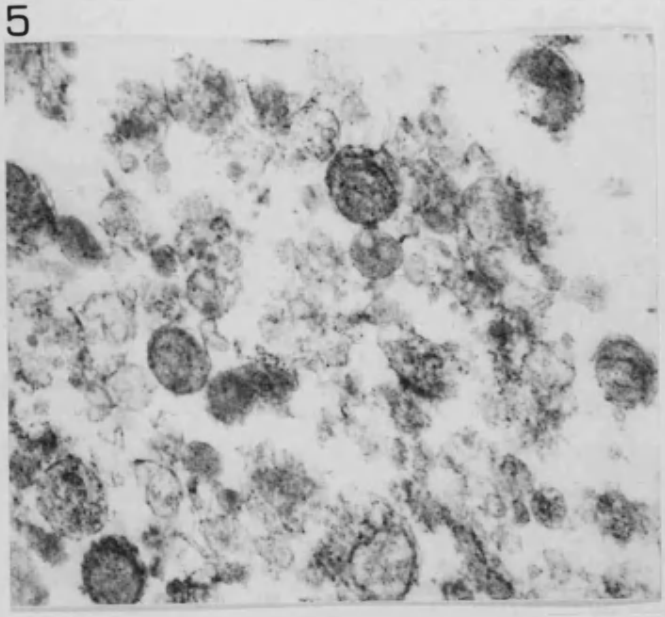
Plates 6 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS. Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none. Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS. Encapsulation:- BSA of suspension after glutaraldehyde fixation. Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

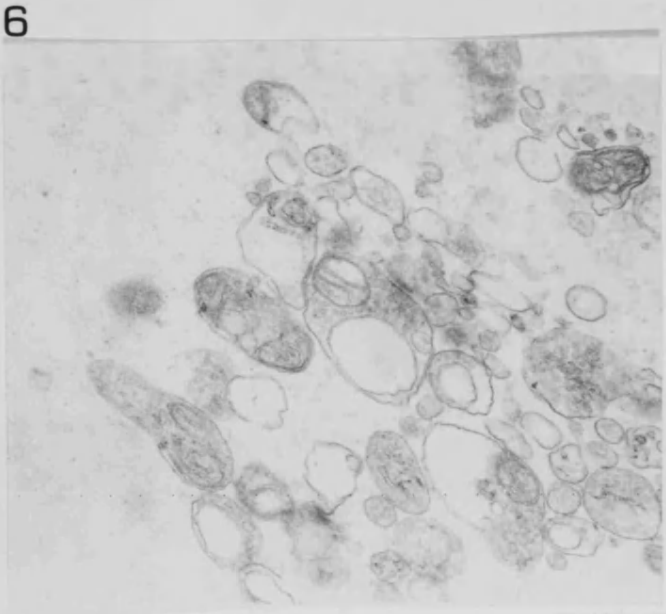
Synaptosomes were not damaged, but the background staining was very high.

5



200nm

6



200nm

Plate 7

Plates 7 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

Encapsulation:- BSA of pellet after glutaraldehyde fixation. Dehydration:- Acetone

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

Synaptosomes were not damaged, but the background staining was very high.

Plate 8

Plates 8 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

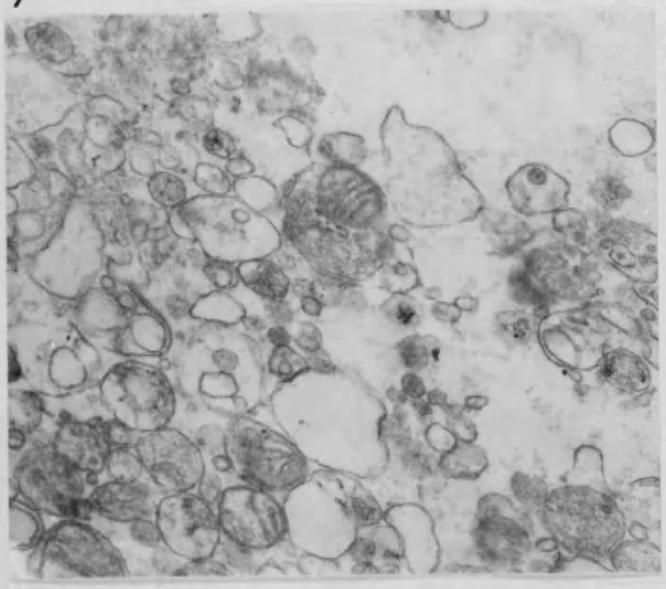
Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

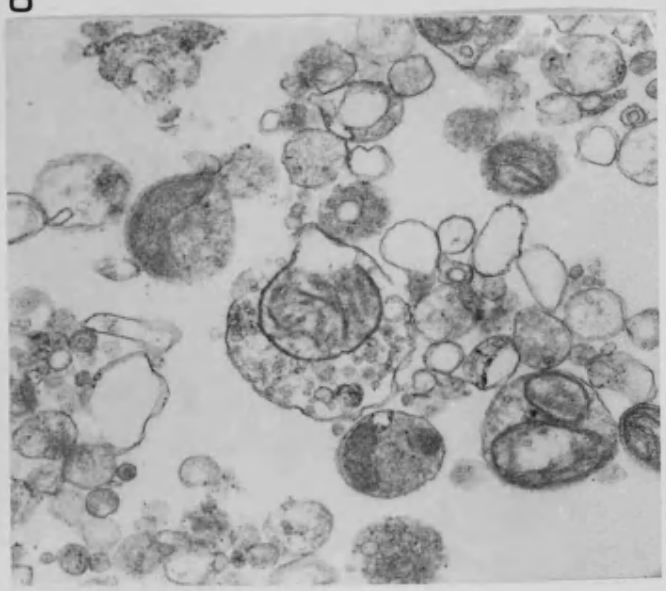
Synaptosomal damage was reduced by forming a pellet after osmium fixation, then treating the pellet as a tissue block.

7



200nm

8



200nm

Plate 9

Plates 9 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- sucrose.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- sucrose.

Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

Synaptosomes were damaged due to centrifugation. The level of background staining was very low.

Plate 10

Plates 10 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- sucrose.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- sucrose.

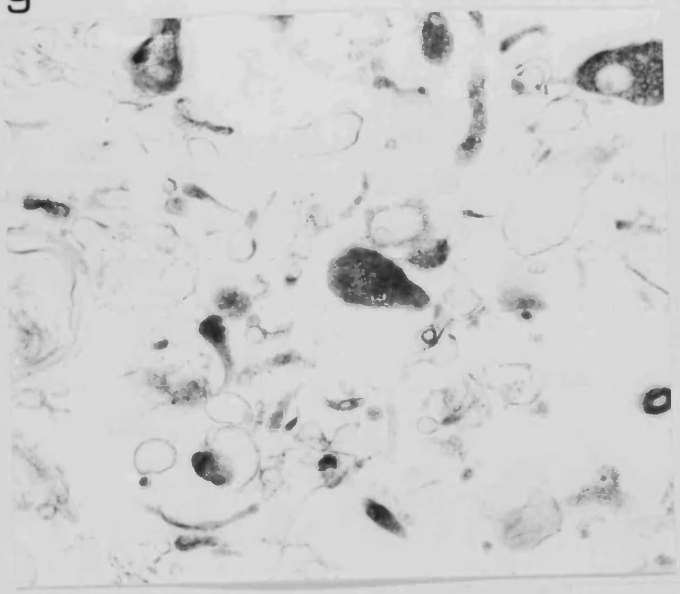
Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- lead citrate.

Results:-

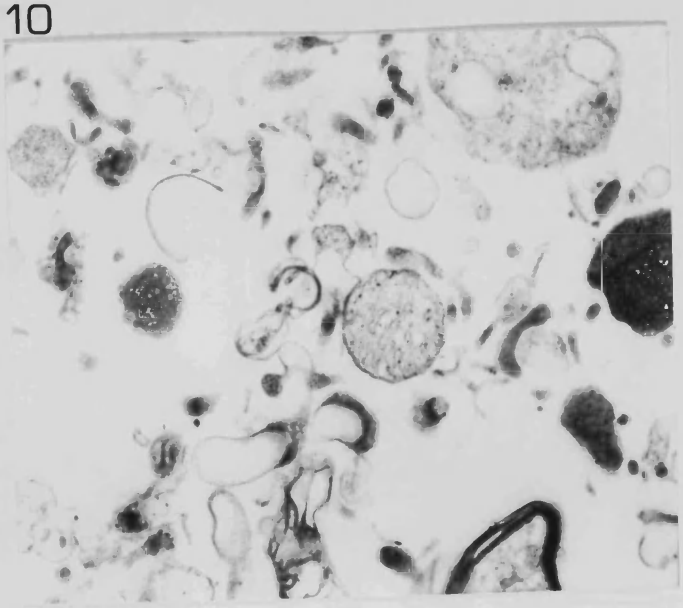
Synaptosomes were damaged as plate 9. The level of background staining was at the same low level as plate 9, showing the staining was due to osmium tetroxide/ glutaraldehyde cross reaction.

9



200nm

10



200nm

Plate 11

Plates 11 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- TBS.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- TBS.

Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- Uranyl acetate/lead citrate.

Results:-

Synaptosomes were damaged. The level of background staining was very low, at a similar level plate 9.

Plate 12

Plates 12 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure).

Immunostaining vehicle:- cacodylate. Concentration of mAb:- none. Secondary probe:- none.

Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C).

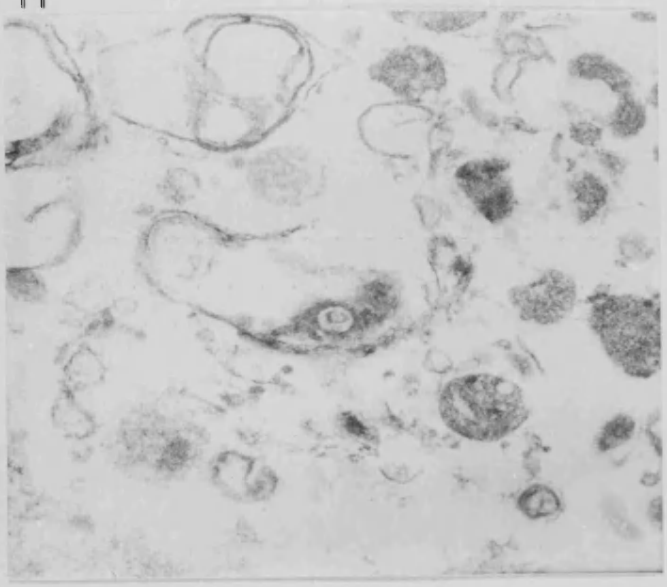
Fixation vehicle:- cacodylate. Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:-uranyl acetate/lead citrate

Results:-

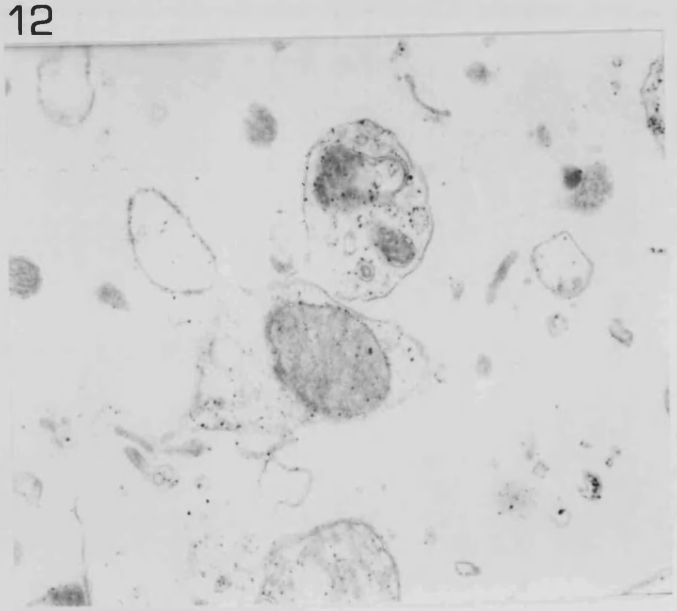
Synaptosomal damage was low, background staining was greater than plate 9.

11



200nm

12



200nm

Plate 13

Plates 13 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- none (prefixation procedure). Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Secondary probe:- none. Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

Encapsulation:- Pellet after osmium fixation. Dehydration:- Acetone.

Embedding medium:- Epoxyresin. Mould:- Eppendorf. Positive stains:- lead citrate.

Results:-

Synaptosomal damage was low. The level of background staining was greater than that found in plate 9. This staining does not reflect chemical cross reaction between uranyl acetate and phosphate, as the level does not differ from plate 2.

Plate 14

Plates 14 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- prefixation. Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Blocking (immunostain):- none.

Secondary probe:- Gold conjugated goat anti-(rat IgG) (2×10^{12} particles, 1ml)

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

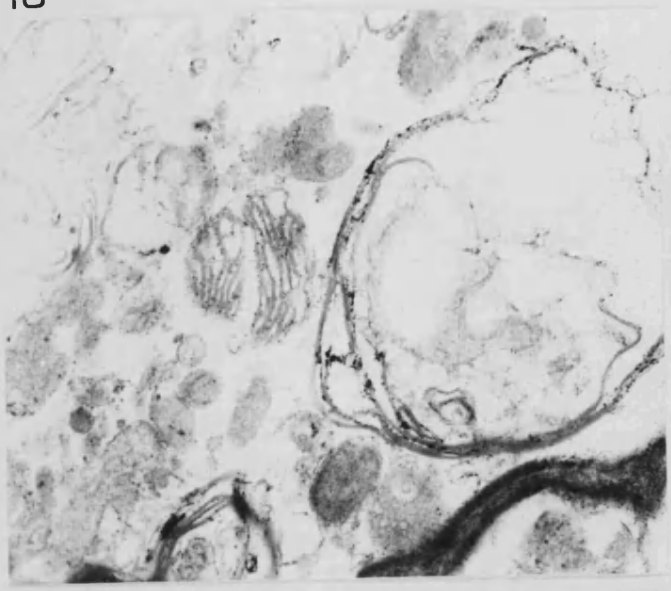
Encapsulation:- none. Dehydration:- Ethanol. Embedding medium:- LR White. Mould: Eppendorf.

Positive stains:- uranyl acetate/lead citrate

Results:-

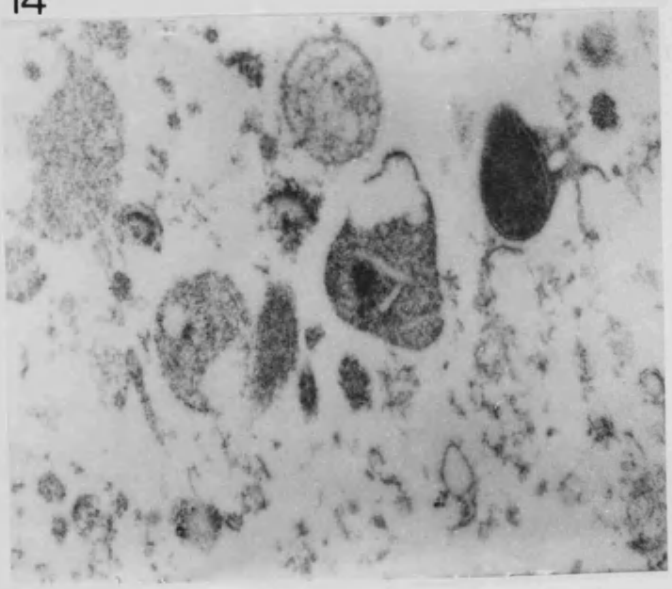
Gold staining was not seen reflecting lack of gold binding or gold dissociation.

13



200nm

14



200nm

Plate 15

Plates 15 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- prefixation. Immunostaining vehicle:- PBS.

Concentration of mAb:- 270 (160 pM), 290 (675 pM), 297 (150 pM), 299 (150 pM). 1ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (2 x 10^{12} particles, 1ml).

Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C). Fixation vehicle:- PBS.

Encapsulation:- none. Dehydration:- Ethanol. Embedding medium:- LR White.

Mould:- Eppendorf. Positive stains:- uranyl acetate/lead citrate

Results:-

Gold staining was not seen reflecting lack of gold binding or gold dissociation.

Plate 16

Plates 16 shows a representative field at the magnification used for analysis (30k).

Specimen:- P2b. Immunostaining:- prefixation. Immunostaining vehicle:- PBS.

Concentration of mAb:- none. Secondary probe:- Avidin-10nm gold (1.5 x 10^{12} particles, 1ml).

Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (16h, 4°C) then osmium tetroxide (1h, 4°C)

Fixation vehicle:- PBS then sucrose for OsO_4. Encapsulation:- Pellet after osmium fixation

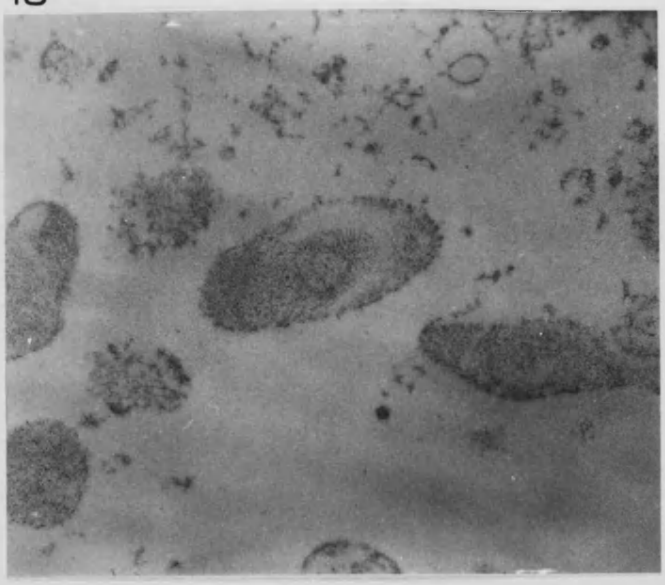
Dehydration:- Acetone. Embedding medium:- Epoxyresin. Mould:- Eppendorf.

Positive stains:- uranyl acetate/lead citrate

Results:-

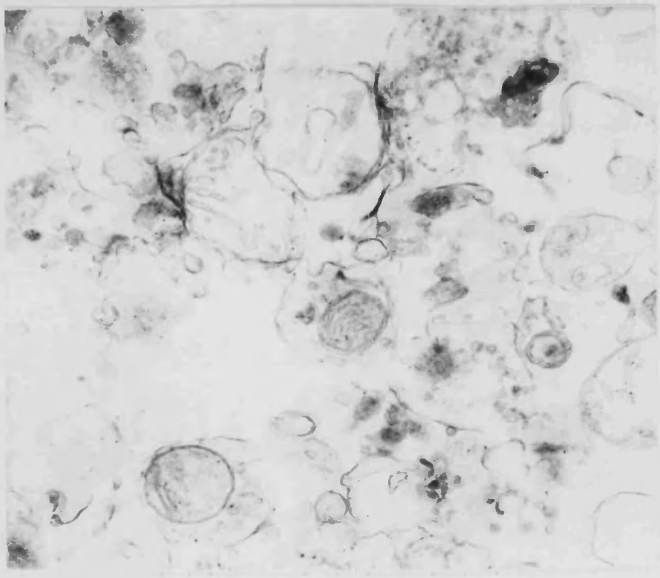
The level of gold staining did not differ from that found in plate 3 where mAb exposure has occurred.

15



200nm

16



200nm

Plate 17

Plates 17 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- none. Blocking (immunostain):- none.

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml)

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- Hot. Mould:- gelatine capsule

Positive stains: - uranyl acetate/lead citrate. Coat:- none. Accelerating voltage:- 80kV.

Plate 18

Plates 18 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- none. Blocking (immunostain):- 1% BSA, 5% goat serum.

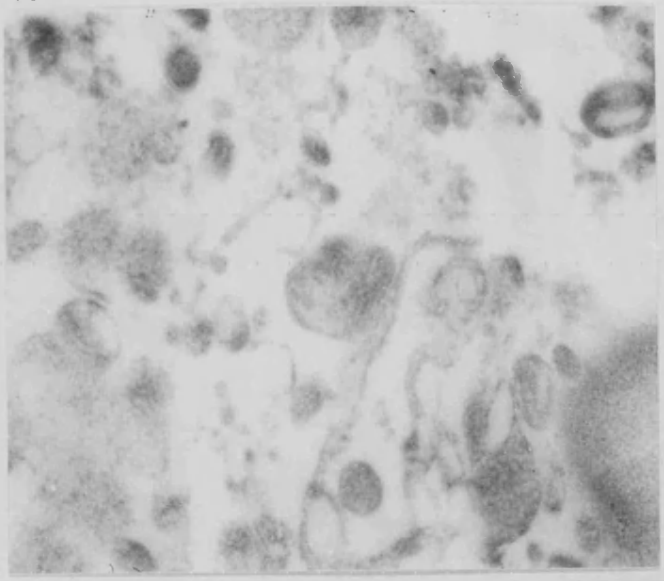
Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- Hot. Mould:- gelatine capsule.

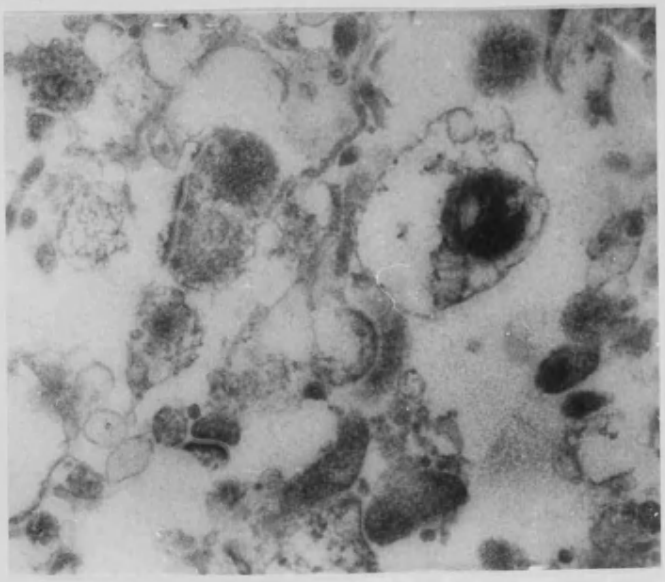
Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 120 kV.

17



200nm

18



200nm

Plate 19

Plates 19 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- PBS, no Triton

Concentration of mAb:- none. Blocking (immunostain):- 0.2% BSA, 5% goat serum.

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- cold. Mould:- gelatine capsule.

Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 80 kV.

Plate 20

Plates 20 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- none. Blocking (immunostain):- 1% BSA, 5% goat serum.

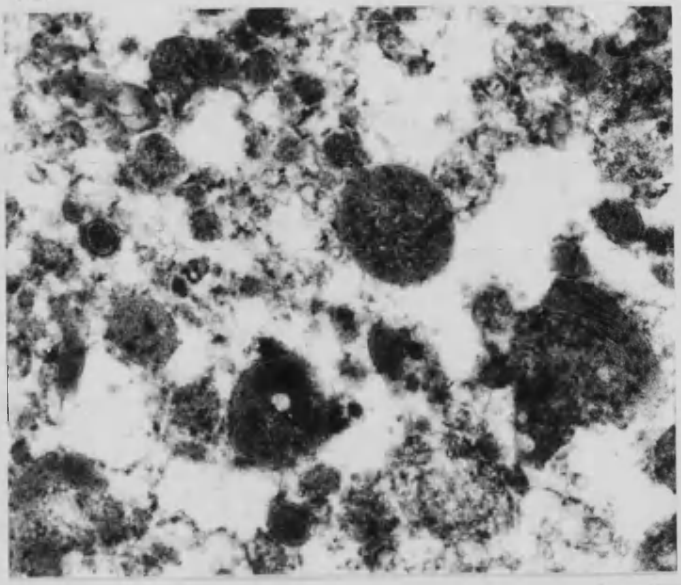
Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- Cold. Mould:- gelatine capsule.

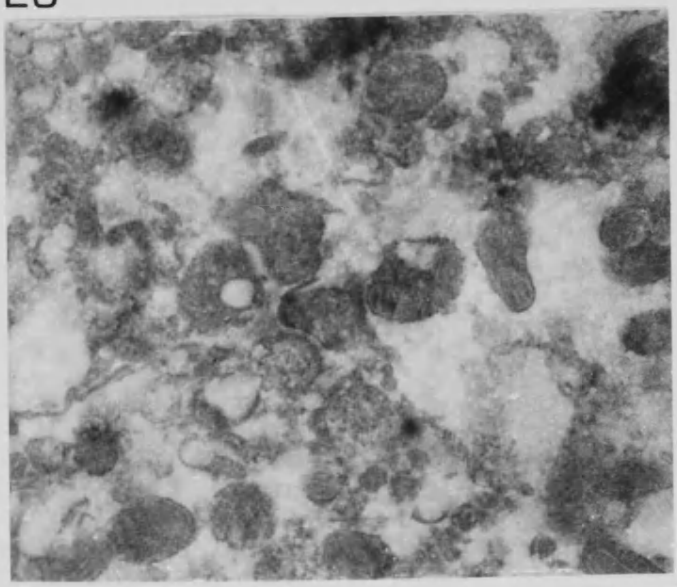
Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 120 kV.

19



200nm

20



200nm

Plate 21

Plates 21 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Blocking (immunostain):- 1% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C).

Fixation vehicle:- PBS. Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- cold.

Mould:- gelatine capsule. Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon.

Accelerating voltage:- 120 kV

Plate 22

Plates 22 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml)

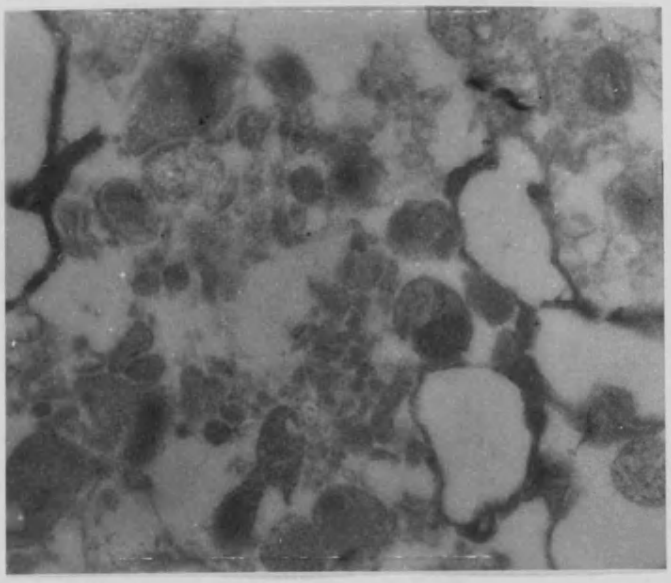
Blocking (immunostain):- 1% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- hot. Mould:- gelatine capsule.

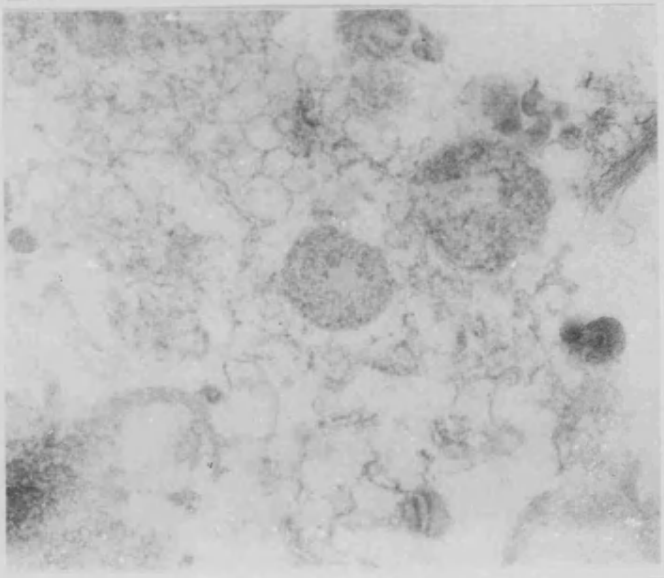
Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 120 kV.

21



200nm

22



200nm

Plate 23

Plates 23 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, no Triton.

Concentration of mAb:- 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Blocking (immunostain):- none.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- hot. Mould:- gelatine capsule.

Positive stains:- uranyl acetate/lead citrate. Coat:- none. Accelerating voltage:- 80 kV.

Plate 24

Plates 24 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- PBS, no Triton.

Concentration of mAb:- 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

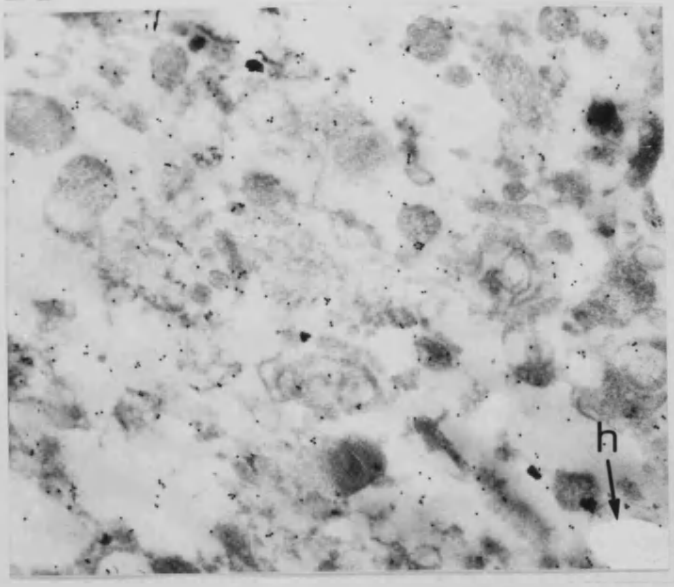
Blocking (immunostain):- 0.2% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- hot. Mould:- gelatine capsule.

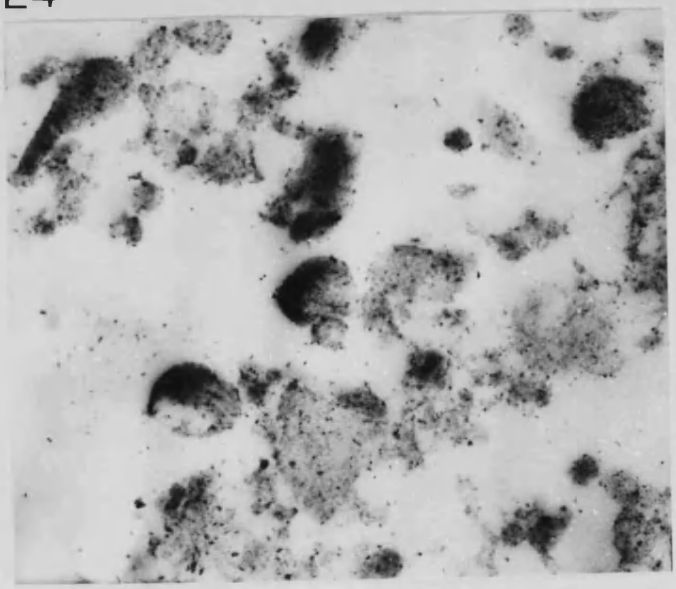
Positive stains:- uranyl acetate/lead citrate. Coat:- none. Accelerating voltage:- 80 kV.

23



200nm

24



200nm

Plate 25

Plates 25 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, Triton.

Concentration of mAb:- 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

Blocking (immunostain):- 1% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- cold. Mould:- gelatine capsule.

Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 120 kV.

Plate 26

Plates 26 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- TBS, Triton.

Concentration of mAb: 270 (800 pM), 290 (13500 pM), 297 (150 pM), 299 (150 pM). 0.25ml.

Secondary probe:- Gold conjugated goat anti-(rat IgG) (1 x 10^{12} particles, 0.2ml).

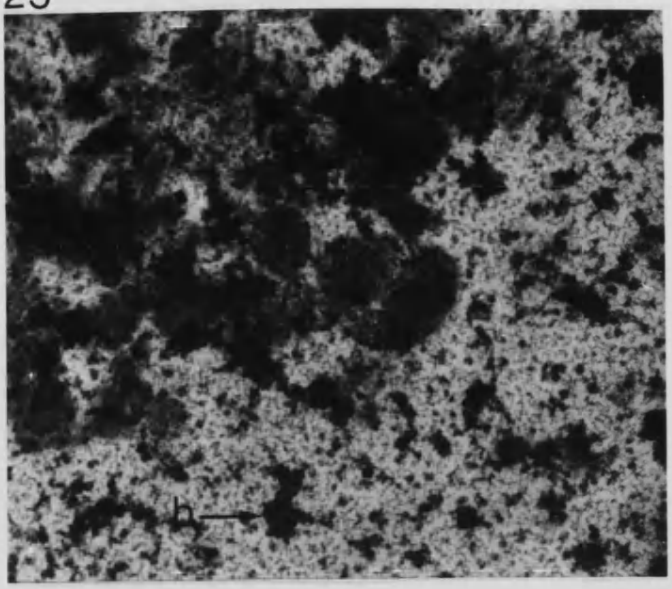
Blocking (immunostain):- 1% BSA, 5% goat serum

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- hot. Mould:- gelatine capsule.

Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 120 kV.

25



26

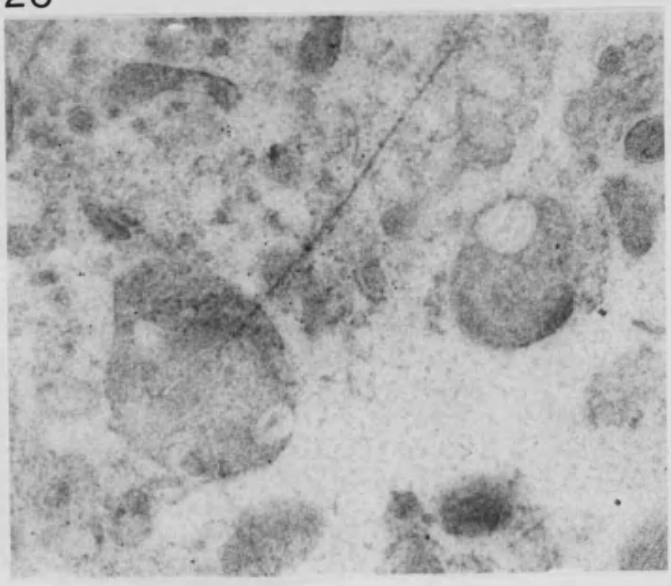


Plate 27

Plates 27 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- PBS, no Triton.

Concentration of mAb:- ANF 2F7F10 (20% v/v).

Secondary probe:- Gold conjugated goat anti-(mouse IgG) (1 x 10^{12} particles, 0.2ml).

Blocking (immunostain):- 0.2% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- cold Mould:- gelatine capsule.

Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 80 kV.

Plate 28

Plates 28 shows a representative field at the magnification used for analysis (30k).

Specimen:- Hb. Immunostaining:- post embedding. Immunostaining vehicle:- PBS, no Triton.

Concentration of mAb:- ANF 2F7F10 (20% v/v).

Secondary probe:- Gold conjugated goat anti-(mouse IgG) (1 x 10^{12} particles, 0.2ml).

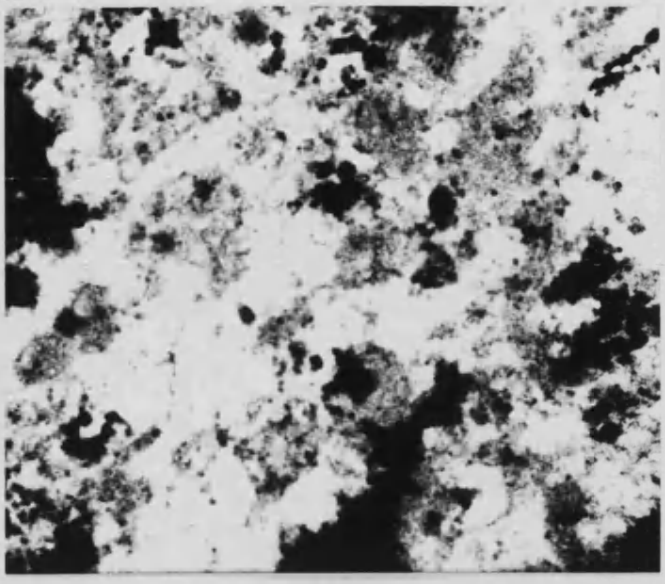
Blocking (immunostain):- 0.2% BSA, 5% goat serum.

Fixation:- Glutaraldehyde (0.1%) and formaldehyde (2%) (1h, 4°C). Fixation vehicle:- PBS.

Dehydration:- Ethanol. Embedding medium:- LR White. Cure:- cold. Mould:- gelatine capsule.

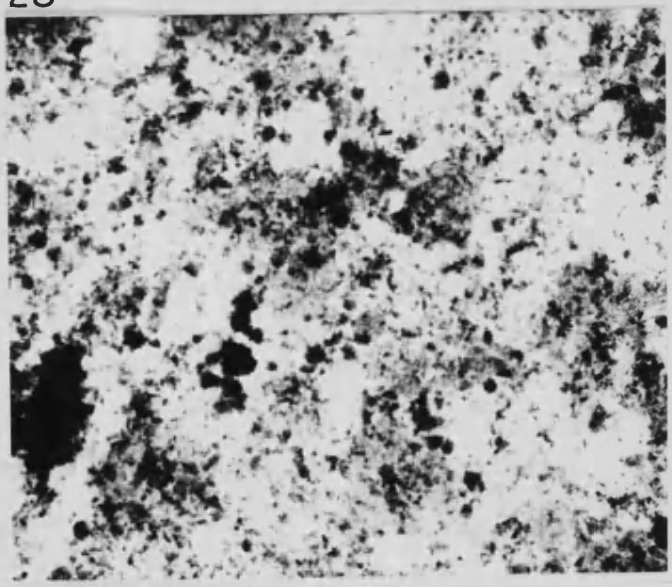
Positive stains:- uranyl acetate/lead citrate. Coat:- piloform/carbon. Accelerating voltage:- 80 kV.

27



200nm

28



200nm

Plate 29 Demonstration of protein estimation by PAGE analysis

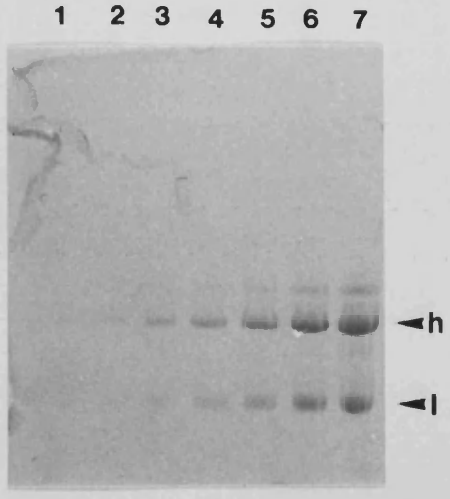
Plate 29 demonstrates the general approach to protein estimation by Phast-PAGE analysis of dilution series. The vanishing point were the light (l) or heavy (h) immunoglobulin chain cannot be seen should be constant for all IgG molecules. This approach avoids protein estimation problems which arise due to stabilizing proteins of different molecular weights (eg BSA).

Plate 30 The determination of an appropriate concentration for the PAGE analysis of P2 membranes

Each PAGE system must be characterized to determine the maximum sample concentration which can be used without overloading. At excessively high concentrations (in this case ≥2mg/ml) the passage of component polypeptides is retarded. The optimal concentration for PAGE analysis, using the minigel system (as section 3.4.1),of P2 membranes (20 μl) was 1.5mg/ml.

lane	concentration (mg/ml)
1	0.25
2	0.35
3	0.5
4	0.75
5	1
6	1.5
7	2
8	3
9	4
10	6

29



30

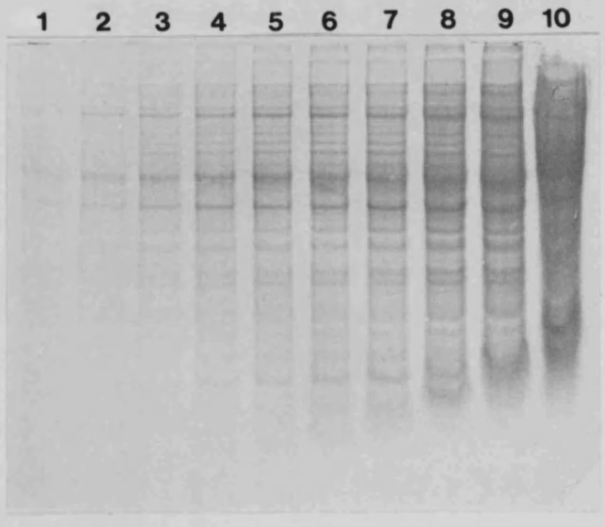


Plate 31 **Immunoblotting using peroxidase conjugated goat anti-rat IgG antibodies; and**

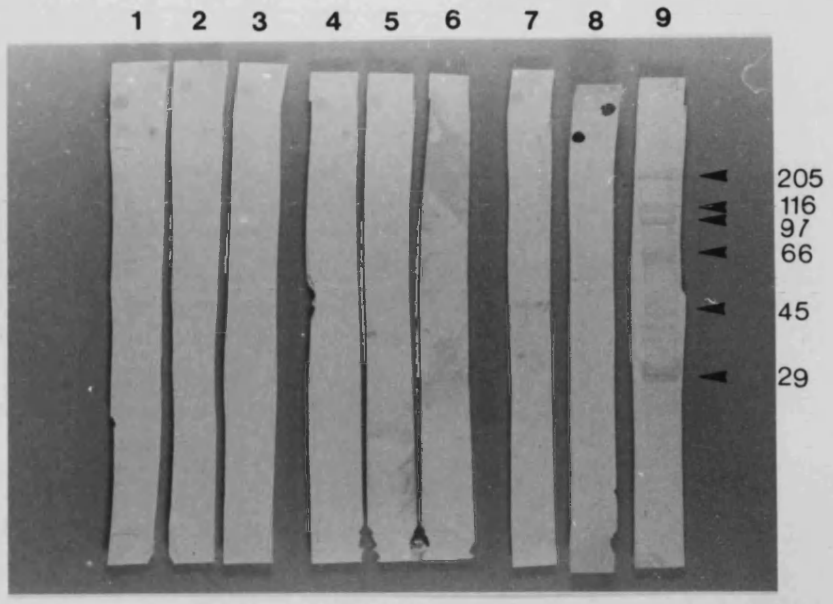
Plate 32 **Immunoblotting using peroxidase conjugated Avidin**

Plates 31 and 32 demonstrate immunoblotting to combined dot blots (figure 4.4a) and PAGE separated P2 membranes. Antibody exposure was performed in 4ml of PBS/Tween, washing was performed in 50 ml of PBS/Tween. Secondary probe concentration is expressed as a percentage of the recommended concentration, the recommended concentration of goat anti-rat IgG was 1/5000. A cocktail of all 4 mAbs was used that 4 μl cocktail contained:-

antibody	volume	final titre (pM)	sufficient to detect
270	0.1μl	40	160 fmol nAChR
290	0.1μl	675	2700 fmol nAChR
297	0.1μl	7.5	30 fmol nAChR
299	0.1μl	7.5	30 fmol nAChR

lane	Immunoblotting		Plate 31	Plate 32
	Primary probe	Secondary probe	Block in PBS/Tween	
1	16μl cocktail	500%	5% w/v milk	none
2	16μl cocktail	100%	5% w/v milk	none
3	16μl cocktail	20%	5% w/v milk	none
4	80μl cocktail	100%	5% w/v milk	none
5	16μl cocktail	100%	5% w/v milk	none
6	3.2μl cocktail	100%	5% w/v milk	none
7	16μl cocktail	100%	none	Avidin/biotin block
8	Protein stain			
9	Molecular weight markers (Sigma MW-SDS-200)			

31



32

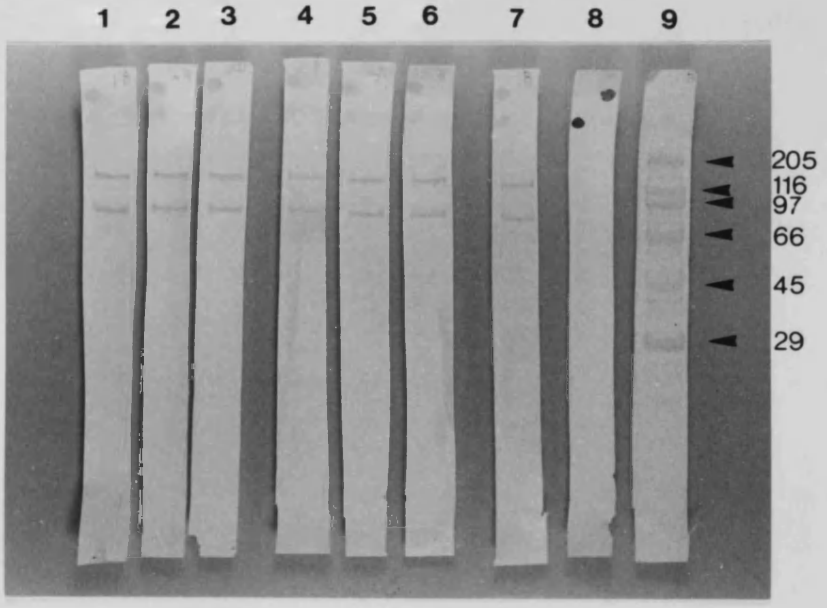


Plate 33 Immunoblotting using [^{125}I]-Avidin

Plate 33 demonstrates immunoblotting to combined dot blots (figure 4.4b) and PAGE separated P2 membranes. Antibody exposure was performed in 4ml of PBS/Tween, washing was performed in 50 ml of PBS/Tween. A cocktail of all 4 biotinylated mAbs was used such that overall titres (of the more dilute antibodies) were the same as plate 31:-

lane	Immunoblotting		
	Primary probe	Secondary probe	Block in PBS/Tween
1	16μl cocktail	11 pM (44 fmol)	None
2	16μl cocktail	44 pM (176 fmol)	None
3	16μl cocktail	176 pM (704 fmol)	None
4	80μl cocktail	44 pM (176 fmol)	None
5	16μl cocktail	44 pM (176 fmol)	None
6	3.2μl cocktail	44 pM (176 fmol)	None
7	16μl cocktail	44 pM (176 fmol)	Avidin/biotin block
8	Protein stain		
9	Molecular weight markers (Sigma MW-SDS-200)		

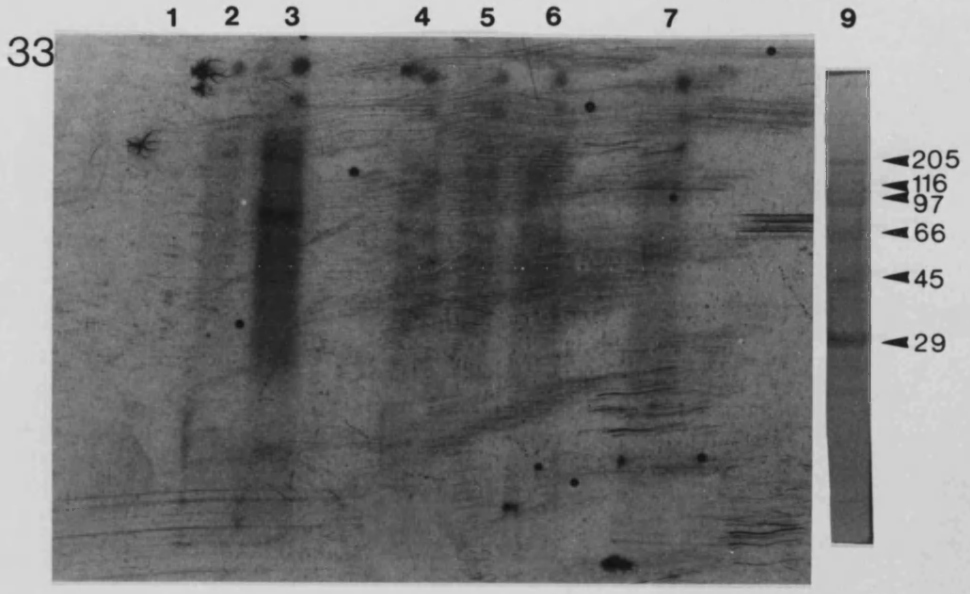

33
1
2
3
4
5
6
7
9
205
116
97
66
45
29

Plate 34 Immunoblotting using peroxidase conjugated goat anti-rat IgG antibodies

Plate 34 demonstrates immunoblotting of PAGE separated material is unaffected by the presence or absence of mAbs. Antibody exposure was performed in 4ml of PBS/Tween, washing was performed in 50 ml of PBS/Tween. The secondary probe was used at the recommended concentration (1/5000). The primary probe was 16μl of a cocktail of all 4 mAbs was used such that overall titres were the same as plate 31:-

lane	Immunoblotting		
	Primary probe	Block in PBS/Tween	Preparation
1	No	5% w/v milk	P2
2	No	5% w/v milk	P2b
3	Molecular weight markers (Sigma MW-SDS-7B)		
4	Yes	5% w/v milk	P2
5	Yes	5% w/v milk	P2b

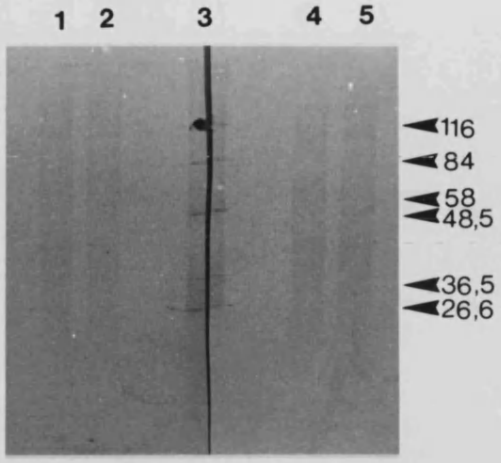
Plate 35 Immunoblotting using [^{125}I]-Avidin

Plate 35 demonstrates immunoblotting of PAGE separated material is unaffected by the presence or absence of mAbs. Antibody exposure was performed in 4ml of PBS/Tween, washing was performed in 50 ml of PBS/Tween. The secondary probe was used at 44 pM (176 fmol). The primary probe was 16μl of a cocktail of all 4 biotinylated mAbs was used such that overall titres were the same as plate 31.

lane	Immunoblotting		
	Primary probe	Block in PBS/Tween	Preparation
1	Yes	Biotin/Avidin	P2
2	Yes	None	P2
3	No	Biotin/Avidin	P2
4	No	None	P2

Molecular weight markers (Sigma MW-SDS-200) were used throughout.

34



35

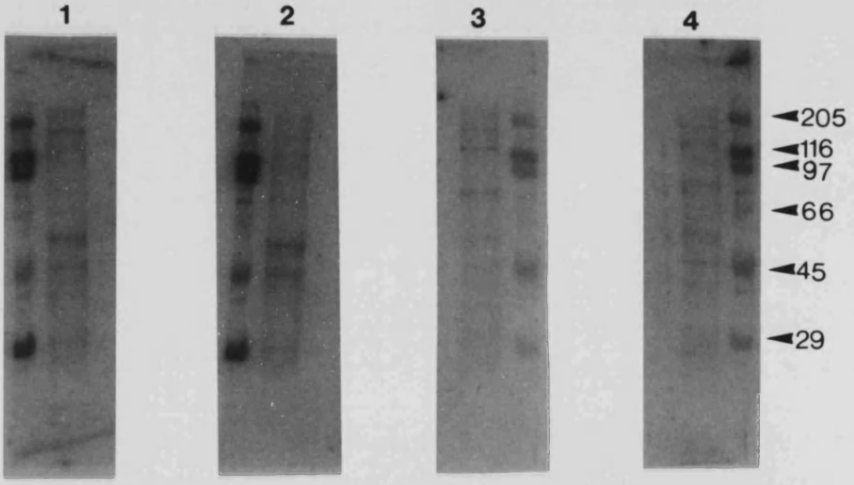


Plate 36 Immunoblotting using peroxidase conjugated goat anti-rat IgG antibodies and mAb 270 alone

Plate 36 demonstrates immunoblotting of PAGE separated material using mAb 270 alone. Antibody exposure was performed in 4ml of PBS/Tween, washing was performed in 50 ml of PBS/Tween. The nitrocellulose was blocked using 5% w/v milk in PBS/Tween. The primary probe was 0.4μl of mAb 270 (titre 160 pM), which was sufficient to detect 640 fmol nAChR. The secondary probe was used at the recommended concentration (1/5000).

lane	1	2	3	4	5	6	7	8
Preparation	Homogenate	S1	P2	F1	F2	F3	F4	F5

Plate 37 Immunoblotting using peroxidase conjugated goat anti-rat IgG antibodies and mAb 297 alone

Plate 37 demonstrates immunoblotting of PAGE separated material using mAb 297 alone (0.4μl of mAb 297, titre 30 pM, which was sufficient to detect 120 fmol nAChR). The procedure and lanes were as described for plate 36.

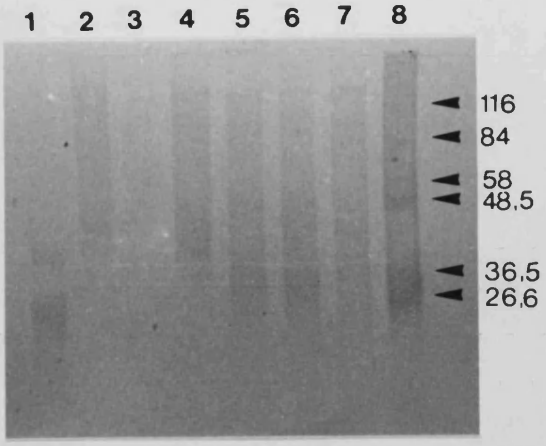
Plate 38 Immunoblotting using peroxidase conjugated goat anti rat IgG antibodies and mAb 299 alone

Plate 38 demonstrates immunoblotting of PAGE separated material using mAb 299 alone (0.4μl of mAb 299, titre 30 pM, which was sufficient to detect 120 fmol nAChR). The procedure and lanes were as described for plate 36.

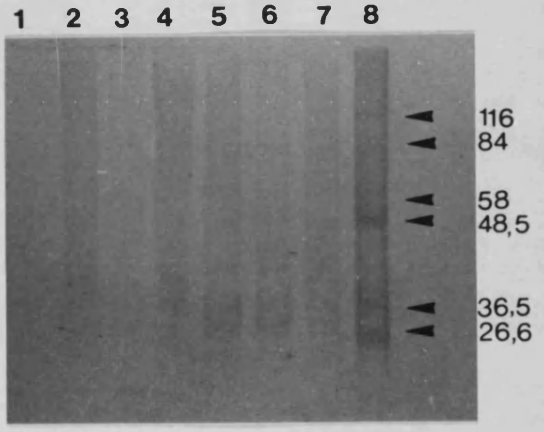
Plate 39 Verification of mAb attachment

The procedure described in section 3.2.4was used to confirm the attachment of mAbs to beads. In this case, mAb 290 (40μl) had been attached directly to cyanogen bromide activated sepharose (150 mg). The beads had been blocked by incubation in 5% w/v milk in PBS/Tween, then incubated with peroxidase conjugated anti-(rat IgG) antibodies (1/5000), before oxidative DAB deposition. The difference was very clear between mAb coated (2) and control (1) beads.

36


1 2 3 4 5 6 7 8
116
84
58
48,5
36,5
26,6

37


1 2 3 4 5 6 7 8
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84
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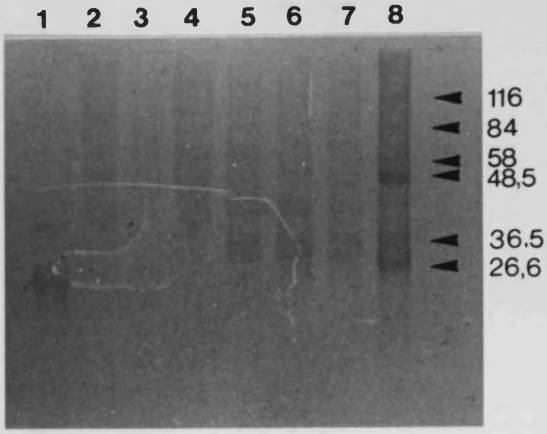
48,5
36,5
26,6

38


1 2 3 4 5 6 7 8
116
84
58
48,5
36,5
26,6

39